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248
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ANIMAL REPRODUCTIVE TECHNOLOGIES AND THEIR POTENTIAL
APPLICATIONS FOR HUMAN FERTILITY PROBLEMS: A REVIEW

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Table of Contents

A. Part I. - Review of Topic Areas	<u>Page</u>
Introduction	1
Embryo Transfer: Historical Prospective	4
Development of Non-Surgical Embryo Transfer Methodology	7
The Potential for the Transplantation of Gonadal Tissue	12
Superovulation Treatments for Supplemental Oocyte Production	15
Maturation of Oocytes with <u>In Vitro</u> Systems	24
The Potential for Sexing Sperm Cells	27
<u>In Vitro</u> Fertilization (IVF)	31
Fertilization of the Ovum by Sperm Cell Microinjection	37
Freezing Mammalian Embryos	38
Frozen Storage of Oocytes	46
The Potential for Frozen Storage of Spermatozoa	49
<u>In Vitro</u> Culture Systems for Embryos	58
Sexing Mammalian Embryos	66
Genetic Engineering - Its Potential for Farm Animals	70
Micromanipulation of Embryos to Produce Multiple Offspring	70
Splitting Embryos to Produce Twins	74
The Production of Chimeric Embryos	82
Inter Cell Mass Transfers Between Embryos	83
Embryonic Cloning	87
DNA (Gene) Injection to Produce Transgenic Animals	91
B. Part II. - Progress and Potential Application of Animal Repro- ductive Technologies to Humans	95
Human Fertility Problems	96
Development of Non-Surgical Embryo Transfer Methodology	99

Cont'd	Page
Potential for Transplantation of Gonadal Tissue	100
Superovulation Treatments for Supplemental Oocyte Production . . .	102
Maturation of Oocytes with <u>In Vitro</u> Systems	104
The Potential for Sexing Sperm Cells	106
<u>In Vitro</u> Fertilization Procedures	108
Fertilization of the Ovum by Sperm Cell Microinjection	111
Freezing and Storage Mammalian Embryos	113
Frozen Storage of Oocytes	115
The Potential for Frozen Storage of Spermatozoa	117
<u>In Vitro</u> Culture Systems for Embryos	121
Sexing Mammalian Embryos	125
Splitting Embryos to Produce Twins	128
The Production of Chimeric Embryos	129
Inter Cell Mass Transfers Between Embryos	130
The Potential for Embryonic Cloning	132
DNA (Gene) Injection to Produce Transgenic Animals	133
Other Reproductive Technologies that May Be Applicable to Humans .	137
C. Part III. - Summary of the Potential Application of Animal Repro- ductive Technologies to Humans (Condensed Format) . .	138
Species x Technology Matrix Summary	142
Time-Scale x Technology matrix Summary	143
Concluding Remarks	144
D. References	146

Introduction

Starting in the decade of the 1970's, advances in reproductive physiology were achieved at a staggering rate. In the late 1970's and in this decade new technology employing recombinant DNA and micromanipulation of genetic material to improve animal productivity also became a reality. It appears that the development of artificial insemination (AI), embryo transplantation and embryo freezing techniques for mammals were just the beginning of a new age in animal reproduction technology. Although the greatest genetic impact on animal improvement originates from the male through AI (Humes and Godke, 1975 and others), animal reproductive technology now offers new opportunities to expand maternal genetics, as well as those from the sire's side (Bradford and Kennedy, 1980; Seidel, 1981, Rutledge and Seidel, 1983; Dickerson and Wilham, 1983; Humes and Godke, 1985). Recent developments in molecular biology and genetic engineering will likely offer a new dimension in research and development for future application to animal reproductive technologies. Important discoveries in reproductive physiology over the years that have made an impact on research and technological advancements in animals and man are presented in Tables 1 and 2.

Table 1. Important Discoveries in Reproductive Physiology That Have Made an Impact on Research and Technological Advancements in Animals and Man prior to 1980

Year	Discovery	Researchers
1782	Use of artificial insemination and effect of cooling spermatozoa in mammals (dogs and other species)	Spallanzani
1799	First human pregnancy reported from artificial insemination	Hunter
1890	First documented embryo transplantation producing offspring (rabbits)	Heape ^a
1949	Using a cryoprotectant to successfully freeze avian and later mammalian semen	Polge & co-workers ^a Polge & Rowson ^a
1959	The first live rabbit offspring produced from <u>in vitro</u> fertilization	Chang ^a
1972	Live offspring from frozen mammalian embryos (mice)	Whittingham & co-workers ^a
1973	First frozen-thawed farm animal offspring produced by embryo transplantation (cattle)	Wilmut & Rowson ^a
1973	Transplantation of a single gene from animals to microbes. Basis for the new recombinant DNA technology	Cohen & Boyer
1978	Dividing embryos to produce genetically identical genotypes (mice)	Moustafa & Hahn ^a
1978	Successful transplantation of ovaries from a female farm animal to another reported (cattle)	Griffin & co-workers ^a
1978	The first documented IVF baby born from autotransfer (humans)	Steptoe & Edwards ^a
1979	Transplantation of a synthesized gene	Gilbert & co-workers
1979	First multiple offspring of farm animals produced by embryo micromanipulation (sheep)	Willadsen ^a

^aDiscoveries cited by first author in the Reference section of the manuscript.

Table 2. Important Discoveries in Reproductive Physiology Which Have Made an Impact on Research and Technological Advancements in Animals and Man prior to 1980 to present

Year	Discovery	Researchers
1981	First IVF farm animal offspring born (cattle)	Brackett & co-workers ^a
1981	Transplantation of a gene from one species to another (mice)	Wagner & co-workers ^a
1982	Using an immunological approach to sexing embryos (rabbits)	Wagner ^a
1982	A practical approach to splitting later-stage farm animal embryos to produce genetically identical twins (cattle)	Williams & co-workers ^a Lambeth & co-workers ^a
1983	Nuclear transplantation in mice embryos by microsurgery and cell fusion (mice)	McGrath & Solter ^a
1984	First frozen-thawed human embryo (IVF) transplant baby born in Australia (humans)	Trounson & Mohr ^a Trounson & Wood
1984	Using an H-Y antibody procedure to successfully sex farm animal embryos (cattle)	White & co-workers ^a Wachtel ^a
1985	First transgenic offspring produced in farm animals (swine and sheep)	Hammer & co-workers ^a
1986	First embryonic clone offspring produced in farm animals (sheep)	Willadsen ^a

^aDiscoveries cited by first author in the Reference section of this manuscript.

Embryo Transfer: Historical Perspective.

The first part of this developing technology actually occurred near the end of the 19th century, when the first live birth of mammalian offspring from embryo transfer (rabbits) was reported in the scientific literature (Heape, 1891). Walter Heape, then a student at Cambridge University, surgically transferred two embryos (4-cell stage) from an Angora doe rabbit to a Belgian hare doe which had been mated with a Belgian hare male. On May 29, 1980 the Belgian hare gave birth to six live offspring, two were albino, long haired Angora transplant offspring originating from the embryos of the Angora donor doe.

Unfortunately, Heape's accounts of embryo transplantation in rabbits in the 1890's lay dormant in the research archives of the Royal Society in London for a number of years. Subsequently, other researchers became keenly interested in mastering this technique in laboratory animals in hopes of studying various aspects of mammalian developmental biology. During progressive decades of the 20th century (starting in the 1930's) embryo transplantation success was reported in laboratory mice, rats, hamsters and guinea pigs, in addition to further embryo transfers in the domestic rabbit.

As interest in this technique grew, other researchers conducted successful transfer experiments with more exotic species such as the Snowshoe hare, wild rabbit, mink, ferret, quokka and wallaby. More recently, embryo transplant offspring have been reported from the gerbil, dog, cat, Bighorn sheep, zebra, Przewalski's horse, Bongo antelope, Indian gaur, eland, llama, water buffalo, Rhesus monkey and baboon. For importance of embryo transfer technology for zoo animals, see the recent review on the subject by Woolf (1986). In 1978, the first in vitro-

TABLE 3. Successful Embryo Transplantation Has Been
Reported in the Following Mammalian Species

Species	Researchers
<u>Laboratory Animals.</u>	
Rabbit	Heape (1891)
Rat	Nicholas (1933)
Mouse	Fekete & Little (1942)
Golden Hamster	Blaha (1964)
Mongolian Gerbil	Norris (1981)
<u>Farm Animals.</u>	
Goat (autotransfer)	Warwick & Berry (1932)
Sheep	Warwick & Berry (1933)
Goat	Warwick & Berry (1944)
Cow	Willetts <u>et al.</u> (1951)
Pig	Kvasnickii (1951)
Mule (donkey ♀)	Allen & Rowson (1972)
Hinny (donkey ♀)	Allen & Rowson (1972)
Horse	Oguri & Tsutsumi (1974)
<u>Other Species.</u>	
Quokka	Tyndale-Biscoe (1963)
Ferret	Chang (1968)
Tammar Wallaby	Tyndale-Biscoe (1970)
Mink	Adams (1975)
Cat	Schrivver <u>et al.</u> (1978)
Dog	Kinney <u>et al.</u> (1979)
<u>Primates.</u>	
Baboon	Kraemer <u>et al.</u> (1976)
Rhesus Monkey (autotransfer)	Marston <u>et al.</u> (1977)
Man (autotransfer)	Steptoe & Edwards (1978)
Baboon (non-surgical)	Pope <u>et al.</u> (1983)
Man (donor to surrogate)	Buster <u>et al.</u> (1984)
Rhesus Monkey (non-surgical)	Bavister <u>et al.</u> (1985)

fertilized human baby was born in England as a result of autotransplantation (embryo placed back into donor) (Steptoe and Edwards, 1978).

However, the first human embryo transplant baby (donor to surrogate mother) was not reported until February, 1984 by Buster and colleagues at the UCLA Medical Center in California.

Prior to the 1930's, little effort was made to adapt embryo transplant technologies to domestic farm animals. The first offspring born following homologous embryo transfer in the domestic sheep or embryo re-implantation in the domestic goat was reported in Texas by Warrick and co-workers (1934). Subsequently, Warrick and Berry (1949) verified live offspring born following homologous embryo transfer and autotransfer in both the sheep and the goat. Although successful methods for producing viable swine embryo transplant offspring were first reported in the early 1960's in Europe and the USA (Hancock and Hovell, 1962; Vincent and co-workers, 1964; Smidt and co-workers, 1965), a Russian scientist has been given credit for producing the first successful embryo transplant offspring in swine (Kvasnickii, 1951). The same year, researchers at the University of Wisconsin were first to report a live calf from surgical embryo transplantation in cattle (Willet and co-workers, 1951). This first calf caused a great deal of excitement among researchers and cattlemen alike.

Success with equine embryo transplantation prior to the 1970's has not been verified in the scientific literature. In 1972, Allen and Rowson in Cambridge, England used surgical collection and transfer techniques to produce the first donkey x mare hybrid offspring (mule) and the first stallion x donkey hybrid offspring (hinny) using donkey recipient females.

In Japan, Oguri and Tsutsumi (1972) were the first to document a non-surgical method for recovering embryos from domestic mares. Using the same basic non-surgical uterine flushing method, Oguri and Tsutsumi (1974) collected 18 equine ova from 29 mares. When 15 of the embryos were transplanted to recipient mares, six pregnancies resulted in embryo transplant offspring. The first non-surgical transfer of an embryo in the USA resulting in a live foal was reported by Vogelsang and co-workers (1978) at Texas A&M University.

Development of Non-Surgical Embryo Transfer Methodology.

The development of non-surgical collection and transfer techniques for applied use in bovine embryo transplantation is now considered one of the major milestones in developing the commercial cattle embryo industry in North America. Historically, Mutter and co-worker (1964) at the University of Kentucky are given credit for being first to non-surgically transfer a bovine embryo and obtain a viable offspring. A single 16-cell embryo was collected from a slaughtered cow's reproductive tract and transferred with a small cannula to a recipient female, which resulted in a live embryo transplant calf.

Prior to 1970, Sugie (1965) of Japan had been accredited with producing the only documented live embryo transplant calf that had been collected and transferred non-surgically. Using specially designed equipment, Sugie collected and transferred 32 bovine embryos to recipient females which resulted in the birth of one Holstein transplant offspring. During the late 1960's and early 1970's many other attempts were made to develop a practical and efficient procedure for non-surgical embryo transplantation in cattle, but with little success.

In July of 1976, a practical procedure for collecting bovine embryos non-surgically using a flexible catheter was independently reported by three different research groups (Drost and co-workers, 1976; Elsdon and co-workers, 1976; Rowe and co-workers, 1976). In addition to non-surgical bovine recovery procedures, a non-surgical embryo transplanting procedure for cattle using a semen straw insemination pipette was reported from Europe the same year (Brand and co-workers, 1976).

To non-surgically collect cattle embryos, a small synthetic rubber catheter (16 to 24 French) is inserted through the cervix of the donor cow (Figure 1) and a special collection medium is flushed through the uterus to harvest the embryos 7 or 8 days after estrus. This collection procedure is relatively simple and can be completed in 30 minutes or less, without harm to the cow.

Following collection, the embryos are transplanted into recipient cattle using a non-surgical transfer procedure that has been refined so that it is now similar to the standard AI procedure used by many cattle producers. Embryos are individually placed in .25 or .50 milliliter semen straws (with an appropriate volume of holding medium) and inserted directly into the uterus of recipient cattle with the standard Cassou AI catheter. Similar to the procedure for AI, this non-surgical method takes 10 minutes, however, with the non-surgical embryo transfer a 7 to 8 day embryo is placed into the uterus of the female instead of semen.

With the development of embryo transfer technology in the mid-1970's, cattle reproduction again entered a new age of technical advancement. Prior to 1973, no more than 20 embryo transplant pregnancies had been produced by researchers, yet by 1976 there were 20 newly established embryo transplant units in North America. In 1976, the basic

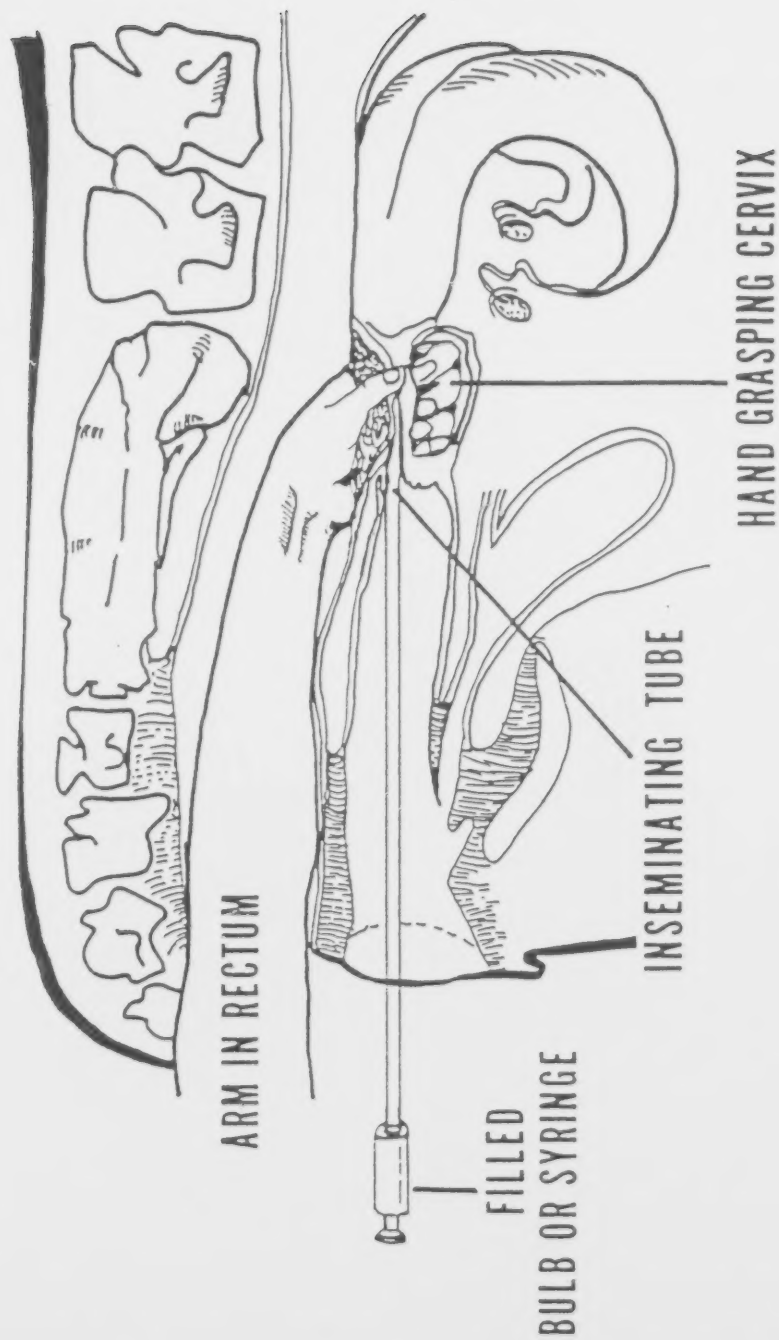


Figure 1. Non-surgical embryo collection method for cattle.

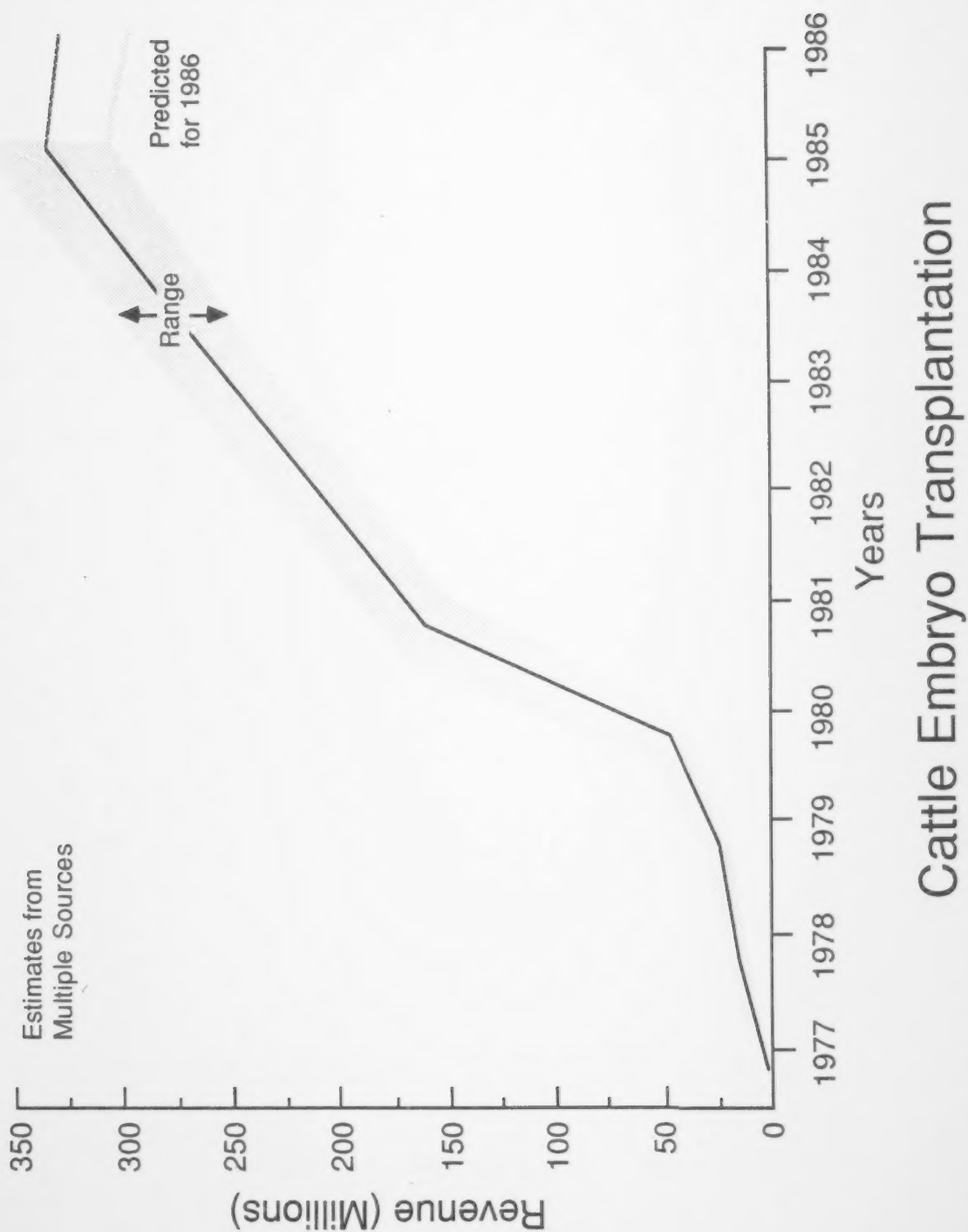
techniques for non-surgical collection and transfer of cattle embryos became available to veterinarians and technicians in the livestock industry.

It has been estimated by Seidel and Seidel (1978) that up to 10,000 pregnancies resulted from cattle embryo transfer in 1978. By the end of 1980, transplant pregnancies were estimated to have reached 20,000 annually (Seidel, 1980a). The number and size of commercial transplant units increased, resulting in approximately 35,000 pregnancies from cattle embryo transfer in 1982. To reduce the cost of producing transplant pregnancies, mobile transplant teams expanded this technology to fit on-the-farm situations, which stimulated nationwide usage of embryo transfer (Figure 2). It has now been estimated that 55,000 or more bovine transplant pregnancies occurred prior to the end of 1984. When the final tabulations for 1985 are in, this figure will likely reach 100,000 or more transplant pregnancies in North America. Projections indicate that these numbers will continue to increase in the years to come.

Following the development of the basic non-surgical techniques, embryo recovery percentages and post-transfer pregnancy rates were initially lower for these procedures than with the standard paralumbar (surgical) approach to transplantation. However, refinement of techniques and technician experience have greatly improved the success rate of these procedures and today has made transplanting embryos a meaningful part of cattle reproductive management, especially for seedstock producers.

Today, with non-surgical collection procedures, one would expect to harvest 75 to 95% of the ova from an adequately stimulated, hormone-

Figure 2



treated donor cow. Usually 60 to 85% of the ova collected from inseminated beef or dairy donors will be fertilized. Often the percent of embryos recovered from single embryo collections of unstimulated donors is usually greater than the percent from superovulated cattle. With an experienced technician, one could expect a 50 to 75% pregnancy rate for viable embryos transferred non-surgically to good quality recipient cattle. For neophytes lacking transfer experience, the results are often much lower. In some cases, no pregnancies result. There is little doubt that the development of non-surgical collection and transfer technology was the largest single contributing factor to the rapid growth of the commercial embryo transplant business in North America today.

Non-surgical collection and transfer procedures are also applicable to the horse (Figure 3), primates and humans, but have not been developed to any extent for practical use in sheep, goats and swine, which is likely due to the lack of anatomical capacity of the rectal cavities (for palpation) these animals. Some progress has recently been reported on developing a repeatable non-surgical embryo recovery procedure in sheep at Texas A&M University (Coonrad and co-workers, 1986). Non-surgical transfer procedures in the mare, however, are usually less successful than transferring equine embryos via the standard surgical method.

The Potential for the Transplantation of Gonadal Tissue.

The ability to transplant gonadal tissue could be of great importance in the production of breeding animals, and possibly could aid in the fight against human infertility. Replacing diseased or injured gonadal tissue with newer (younger) gonadal tissue would aid in the

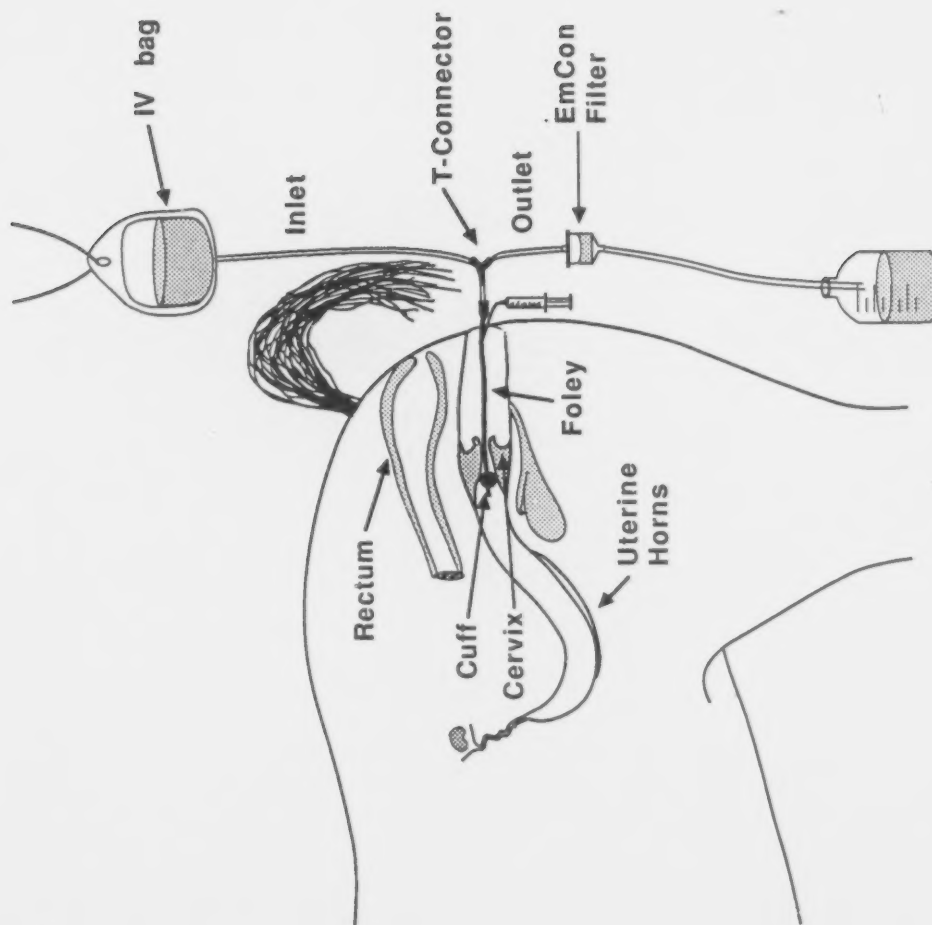


Figure 3. Non-surgical embryo collection procedure for the horse.

correction of some human infertility problems. This is not a new idea for scientists, but little has been done in this area over the years due to the concern for host tissue rejection with gonad heterotransplants. Uncontrollable factors in an animal's life such as age, sickness and physical injury may render a productive animal useless as a breeding female. Successful ovarian transplantation would allow continued gamete production from ovaries of animals from genetically superior bloodlines.

Successful ovarian tissue transplantation has been reported in the mouse (Parrott, 1960; Parkening et al., 1984 and others), rat (Ingram and Krohn, 1956), hamster (Parrott, 1959) and rabbit (Betteridge, 1970). Some success with various approaches to ovarian tissue transplantation has been reported with the sheep (Baird et al., 1968; Binns et al., 1969 and others), swine (Binns et al., 1969) and cattle (Griffin and Randel, 1978; Acosta et al., 1982), however, no results have been reported for ovarian transplantation in the horse.

In the 1950's it was established that the success of tissue transplants depended on the histocompatibility between the tissue donor and the recipient. However, researchers at Texas A&M University (Griffin and Randel, 1978; Acosta et al., 1982) gained attention from researchers when they reported successful ovarian heterotransplants between unrelated cattle, without determining donor-recipient histocompatibility prior to transplant surgery. In both studies ovaries from mature females were transferred to the uterine horns of prepubertal females. Griffin and Randel (1978) also reported that the transplanted ovarian tissue was capable of responding to exogenous gonadotropins. Although no explanation has been given for this success in cattle, it has been suggested that ovarian tissue may be characterized by immunological

privilege over other body tissues (Billingham and Parkes, 1955; Ingram and Krohn, 1956).

Although successful ovarian heterotransplants have not been reported for humans, one has to wonder if it would be possible with the new immunosuppressant drugs (e.g. cyclosporine) now available in human medicine. For women with oviductal problems, the ability to transplant ovaries into the woman's uterus for ovulations during subsequent cycles, certainly deserves further evaluation (Beyth and Folishuk, 1979). Silber (1978) has reported successfully transplanting of a human testicle from one human twin to the other to treat anorchia. The future in this area of research appears to be promising.

Superovulation Treatments for Supplemental Oocyte Production.

Ovarian hyperstimulation of potential animal embryo donors is accomplished by treating the female with exogenously administered hormones (primarily with gonadotropins) that stimulate growth of supplementary follicles on each ovary, most of which will mature and ovulate as a single egg (ovum), then available for ovulation. This is an important part of the overall oocyte production procedure, since most larger mammals (including the human) usually only ovulate one ovum per estrous cycle. Gonadotropins administered to most polyovulatory species will usually increase the number of ova over that which are usually produced during one estrous period. This technique (known as superovulation) allows multiple embryos to be collected from monovulatory species and increased oocyte production from polyovulatory animals. The potential for oocyte production from mammals with superovulation procedures is clearly illustrated in Table 4.

Table 4.
Estimated Number of Oocytes in the
Ovaries at Birth

Mammal	Total Oocytes
Rat	40,000
Dog	700,000
Sheep	40-80,000
Cow	150-297,000 ¹
Sow	500,000
Goat	60-100,000
Horse	60,000
Human	200-400,000

¹Cow 12 years of age still has 2,500 to 24,000 oocytes remaining in her ovaries.

The two primary gonadotropic agents for inducing ovulation in domestic species are follicle stimulating hormone (FSH) and pregnant mare's serum gonadotropin (PMSG). Other lesser known gonadotropic agents have been used to superovulate mammals, but these have been used more for experimental purposes or to replace either FSH or PMSG in superovulation treatment schedule.

PMSG. The first procedures for preparing the PMSG hormone preparation for experimental use were reported in the 1940's (Goss and Cole, 1940; Rimington and Rowlands, 1944). The experimental use of crude PMSG preparations for induction of estrus and ovulation in anestrus sheep was first reported in the early 1930's (Cole and Miller, 1933). The first evaluations of PMSG for inducing a superovulatory response in cattle were reported by Hammond and Bhattacharya (1944), Dowling (1949) and Rowson (1951). In laboratory animals, Rowlands (1944) first reported the use of the PMSG followed by human chorionic gonadotropin (HCG) for superovulating immature rats. Several years later, PMSG was shown that it was an effective agent for superovulating laboratory mice. In rabbits, Hafez (1961) established PMSG dose schedules to obtain optimal superovulation in the doe. Subsequently PMSG has been used to stimulate follicle development in many other animal species large and small, but is not used for this purpose in the horse and human.

This equine origin gonadotropin-like substance has long been known for its potent ovarian stimulatory ability in mice, rats, rabbits, sheep, goats, swine and cattle. Commercial harvesting of PMSG usually takes place between 40 and 100 days of equine pregnancy, with peak gonadotropic activity per ml of serum occurring in mares between days 60 to 80 of gestation. The potent stimulatory capacity of exogenous PMSG

has been attributed to its FSH-like and LH-like properties and its extended half-life in the bloodstream of the treated animals. The gonadotropic activity is believed to be primarily FSH-like, with the FSH:LH ratio of each harvested batch being of major concern as to its potential for stimulatory activity.

When available, PMSG has been used extensively for superovulation of donor cattle in research procedures as well as in commercial embryo transplant units. In fact, in most commercial units it has been the agent of choice at one time or another. The popularity of PMSG, as a gonadotropic agent, stems from its high level of potency and its ease of administration when compared with other short-acting stimulatory agents.

Although various dose levels and combination schemes using PMSG have been evaluated over the years, a single intramuscular dose ranging from 1,200 to 3,500 IU has been reported to be most effective for superovulation of donor cattle for embryo collection. Research has shown that dose levels of 4,000 IU or greater given to donor cattle many times results in a negative or variable stimulatory effect that often include ovarian hypertrophy, poor ovulatory response and increased percentage of unfertilized ova at collection. For a review of PMSG used as a superovulatory agent in farm animals see reviews by Foote (1970), Gordon (1975) and Betteridge (1977).

Today commercial transplant units that still incorporate PMSG as a part of their superovulation program use various single intramuscular doses ranging between 1,800 to 3,200 IU, with 2,200 being one of the most often used dose levels for donor cows. There is some indication that older, heavier cattle require slightly larger doses of PMSG and there appears to be some degree of variability between breed types.

Although PMSG is recommended for use via an intramuscular route, some have claimed success with its use following subcutaneous or intravenous administration. Since increased environmental temperatures apparently alter the PMSG carrier protein complex when in solution form, it is recommended that it be kept on ice up to a few minutes prior to injection.

Major drawbacks most often cited for the use of PMSG in bovine embryo transfer procedures include:

- (1) Variability in individual cow response to the same dose.
- (2) No control over the level of stimulation once it is administered to the donor animal.
- (3) Over stimulation of the ovarian tissues in many donor cows.
- (4) An inconsistent ovulation pattern with its use in cattle. This appears to be less of a problem with other animals.
- (5) Unfertilized ova or poor quality embryos have been attributed to its use.
- (6) Possibility of refractoriness with repeated use on the same donor animal.
- (7) Variability in gonadotropic activity between different batches when purchased.
- (8) Lack of commercial availability on a regular basis.

It should be noted that although many positive claims as well as a multitude of negative side effects have been attributed to PMSG over the years, however, not all of these indications have been proven by controlled scientific experimentation.

FSH. In recent years, FSH-P (Follicle Stimulating Hormone — Pituitary, Armour Company), a commercially available product, has been shown to be an effective gonadotropic stimulating agent in cattle, sheep and more recently goats. This lyophilized product is a partially purified follicle stimulating hormone which has been obtained from

selected pituitary glands of domestic animals (e.g. pigs) at slaughter. The assigned potency is designated as 1 mg of FSH-P being equivalent to one Armour Unit of pituitary follicle stimulating hormone.

Proper use of FSH in a superovulation schedule for donor cattle has now been shown to be as effective in commercial transplant units as PMSG. FSH has also been shown to be an effective stimulatory agent for sheep and goat donor females. In fact, many of the most successful transplant stations in the United States and Canada now use FSH as the agent of choice for bovine superovulation in place of PMSG. Since the half-life of exogenous FSH is believed to be 3 to 6 hours in the blood donor females, twice daily injections are often administered (am and pm) to maintain endogenous circulating levels for efficient stimulation of follicular growth. In review of the information available there appears to be as many dose modifications of this FSH-injection schedule as there are active transplant units, with no one scheme proven conclusively to be superior over another. This modified FSH treatment schedule has, in some cases, become the "trade-mark" of individual embryo transplant units.

FSH Combined with LH. In a continued effort to improve superovulation in donor cattle various combination treatment schedules using FSH-P and LH have been developed and are presently being evaluated at commercial transplant stations. By adding a commercial LH preparation to the FSH stock solution during the treatment schedule, an attempt is made to insure final growth and maturation of the developing follicles under FSH stimulation. LH ranging from 1 to 20% of the FSH-P (v/v) added to the daily injections has been indicated as giving successful superovulation responses in both dairy and beef donor animals. It

should be noted that some commercial units have been reporting respectable embryo transplant pregnancy rates without the use of LH in combination with FSH during superovulation procedures.

Other Agents. In early days of bovine embryo transfer, other substances with varying degrees of gonadotropic activity were used as a part of the basic superovulation schemes for cattle. One of these agents, HCG (Human Chorionic Gonadotropin) was often used in conjunction with PMSG to enhance final follicular growth and to induce ovulation in donor animals.

In some cases, various estrogen preparations were administered in conjunction with or following ovarian stimulatory agents in attempts to intensify behavioral estrus responses and possibly to induce a controlled LH peak in donor animals. Also, when GnRH (Gonadotropin Releasing Hormone) became available for research it was evaluated for its endogenous LH-releasing capacity following ovarian stimulation.

Although some of these agents were more effective than others, the need for such agents diminished when prostaglandins became available to induce luteolysis during the treatment scheme. Following the administration of prostaglandins, reduced circulating progesterone levels allows for the endogenous release of LH, which resulted in final follicular development (maturation) and ovulation in these donor animals.

Over the years, various crude pituitary extracts and several partially purified commercially prepared pituitary extracts have been tested for gonadotropic activity in donor cattle, however, these extracts showed little consistency in their stimulatory response and are presently not being used to any great extent in the commercial transplant industry.

In contrast, a commercially available source of equine pituitary extract (EPE) has been reported to show favorable success rates when attempts were made to induce superovulation in cattle. In a recent study, daily dose levels of EPE (Pitropin) ranging from 250 to 1,000 FHR Units (Fevold-Hisaw Rat Units) were administered to beef cattle on days 12, 13, 14, 15 and 16 of the estrous cycle, which resulted in 83% of the animals having 12 or more corpora lutea (CL) at necropsy.

Prostaglandins. In the early 1970's prostaglandin- F_2 alpha (PGF_2 alpha) was shown by various research groups to have luteolytic capabilities in cycling cattle, sheep, goats and horses when administered during the mid-diestrous phase of the estrous cycle. Without the use of prostaglandins to regress functioning luteal tissues in the cow, the gonadotropin treatment must be started on days 15, 16, or possibly 17 of the estrous cycle to take advantage of natural luteolysis in the female. However, with the use of a luteolytic agent the superovulation treatment schedule can begin any time between days 7 and 15 of the estrous cycle. Prostaglandins offer the advantages of increased overall effectiveness of the various gonadotropic agents, decreased variability in the interval from the onset of treatment to behavioral estrus and latitude in treating donors on different days (luteal phase) of the cycle to better fit available cyclic recipients or to help disseminate the daily work load of the embryo transplant team.

Although a single 25 mg intramuscular dose of PGF_2 alpha (Lutalyse, Upjohn Company) is generally recommended for induced luteolysis in cycling cattle, single doses of 25, 30, 33.5, 35, 37.5, 40 and 50 mg of PGF_2 alpha have been indicated as being effective for this purpose during superovulation procedures with donor cows and heifers. When

administered as a single dose, prostaglandins are usually administered 24, 48, 60 or 72 hours after the onset of the gonadotropin treatment. Today many commercial transplant units administer a single intramuscular dose of 25 to 35 mg of PGF₂ alpha 48 hours after the initial treatment with the stimulatory agent. Others report the use of a multiple dose of PGF₂ alpha during superovulation procedures on donor animals. Researchers at Colorado State University have shown that 25 mg of PGF₂ alpha at 48 hours followed by a 12.5 mg dose 12 hours later gives excellent results on both cows and heifers.

Results show that cloprostenol (Estrumate), a potent prostaglandin analogue, at dose levels of 500 or 750 mcg given to donors can effectively replace PGF₂ alpha in the superovulation procedure, without altering the stimulatory response. After treatment with PGF₂ alpha or cloprostenol, the donor animal predictably exhibits standing estrus in 48 hours or occasionally at 72 hours following the prostaglandin treatment. Although superovulation treatments incorporating prostaglandin can be initiated any time between day 6 and 15 of the estrous cycle, there is some evidence that starting with the gonadotropin treatment schedule on days 9, 10 or 11 of the cycle will give the most consistent results with donor animals.

In the opinion of these authors, a successful embryo transplant unit must include prostaglandins in various superovulation schedules to obtain optimal embryo recovery rates from donor cows. It should be noted that even with using prostaglandins in conjunction with various superovulatory agents, 5 to 15% of the treated donor animals generally not exhibit standing estrus during or following treatment. In these cases, it is recommended that the donor animal be inseminated when the

female finally does exhibit estrus, since one or more embryos can be recovered following this breeding.

Maturation of Oocytes with In Vitro Systems.

With recent advancement in the injection of genes into activated ova of mammals and in vitro fertilization methodology in farm animals, there is an increased need to develop efficient oocyte maturation and in vitro culture procedures for oocytes. When oocytes of mammals are released from ovarian follicles at the germinal vesicle stage, they usually have the ability to develop in vitro to metaphase of the second meiotic division (see review by Donahue, 1972). Iwamatzu and Yanagimachi (1975) have indicated that this maturation will only occur after the oocyte has developed a certain size (e.g. hamster), which appears not to be dependent on the actual size of the encompassing layers of the respective follicle.

A review of the scientific literature reveals that fertilization of mammalian oocytes matured in culture is usually very low. Furthermore, very few live offspring have been reportedly born from oocytes matured in culture, fertilized and transferred to recipient females. In the more recent literature, Cross and Brinster (1970) have produced live offspring from this approach in the mouse and Thibault (1972) has made this claim in the rabbit. Recently, success with maturation in culture has been reported with porcine and ovine oocytes in Cambridge, England (Cheng and co-workers, 1986).

The reason the lack of oocyte competence is not clear, however, Thibault (1972) has suggested that sperm head fails to decondense to form the male pronucleus in rabbit oocytes that had been previously

matured in culture. He proposed that the oocytes matured in culture may lack the presence of a male pronuclear growth factor (MPGF) substance, which is thought to originate from granulosa and/or thecal cells. This MPGF substance is believed to be transferred to the oocyte in vivo near the time of ovulation. If this was the case with all mammals, it would suggest that oocyte competence is dependent on maturational influence derived from the cytoplasm of the oocyte.

In laboratory species, it has long been known that the fertilization capacity for ovulated oocytes is greater than that of follicular-derived oocytes obtained at surgery or from excised ovaries.

It should be noted, however, that The oocyte maturation model for the mouse appears to be different than that of humans, since greater than 50% of the oocytes collected from a normal human ovary at laparotomy are capable of fertilization when exposed to good quality human spermatozoa in vitro.

Evidence now available indicates that nuclear maturation alone is not sufficient for oocyte fertilization to occur. A certain maturation process in the ooplasm and loosening of follicular cells around the oocyte are also needed in the process. It is well known that loosening of the follicular cells in culture can be induced by the addition of FSH to the culture medium. In contrast to earlier studies, co-culturing oocytes with follicular cells tends to enhance their in vitro maturational abilities (Fulka and Motlik, 1980; Moor and co-workers, 1980). In a more recent study, Staigmiller and Moor (1984) used a modified TCM-199 medium supplemented with 10% fetal calf serum, FSH, LH and estradiol-17 beta to enhance maturation of sheep oocytes. Using this modified culture medium, it was reported that after 24 hours in

granulosa cell co-culture 42.6% of the ovine oocytes developed through normal embryonic stages following fertilization. However, using porcine oocytes, this approach had not been successful in earlier experiments from the same laboratory.

In cattle, Ball and co-workers (1983) have shown that FSH and cAMP improved the quality of in vitro matured bovine oocytes. Hensleigh and Hunter (1983) also demonstrated that FSH, as well as HCG, could increase the percentage of mature bovine oocytes following culture. In the latter study, Eagle's MEM with 10% fetal calf serum with 1 mcg/ml of FSH resulted in maturation of 54% of the oocytes compared with only 18% successful maturation in the control oocytes. The addition of HCG to the medium also caused a significant increase in oocyte maturation over the control, giving a success rate of 39%. It has been previously postulated that LH and HCG (LH-like in nature) are involved in the removal of the maturation inhibitory substances that are found in the fluids of the larger follicles prior to ovulation. For an excellent review on bovine oocyte maturation see Leibfried and First (1979).

In a recent report, Desjardins and co-workers (1985) have reported successful maturation of equine oocytes in vitro using a modified Krebs-Ringer bicarbonate medium supplemented with 10% fetal calf serum. Culturing the oocytes in tubes for 12 to 96 hours at 37°C, 62% of the oocytes resumed meiosis and exhibited germinal vesicle breakdown (after 12 hours in culture) giving the characteristic pattern of events associated with fertilization.

Although it has been shown that oocytes can be matured in vitro, as determined by specific nuclear and cytoplasmic events, subsequent development after fertilization (either in vivo or in vitro) of the

matured oocytes has not, until recently, been a consistent event. Specific regulating factors which are present in vivo apparently are lacking when the oocytes are placed in a present day in vitro culture system. Developing efficient oocyte maturation and in vitro fertilization methods for virtually all mammals studied have been very elusive. It is indeed interesting that the exception in this case is the human, where oocyte maturation and in vitro fertilization have become common place in in vitro fertilization (IVF) laboratories.

The Potential for Sexing Sperm Cells.

This area of experimentation has been one of the most controversial of all the areas of reproductive physiology research. Procedures and recipes for selecting the sex of offspring in humans at conception has been passed down for generations in "wives" tales, and are often found in written collections of folklore. Many publications are available in the scientific literature describing different methods evaluated for separating X- and Y-bearing spermatozoa in mammals. Results reported by many of these initial papers appear to be very encouraging, but attempts to duplicate these experiments in the same laboratories or in those of others have proven to be discouraging, and most often nonrepeatable.

Some of the more often cited methods proposed for sexing spermatozoa include: centrifugation (Lush, 1925; Schilling, 1966), sedimentation (Bhattacharya, 1966; Schilling, 1971), separation by electrophoretic methods (Koltzoff and Schroder, 1933; Gordon, 1957; Servinc, 1968; Hafs and Boyd, 1974) and alterations in atmospheric pressures (Kiddy and Baily, 1973). For additional details on these approaches and others, see reviews by Kiddy and Hafs (1973), Beatty (1974) and

Betteridge (1978). After evaluating the scientific literature, most scientists agree that the results reported are not very consistent.

Another sexing method using bovine serum albumin (BSA) to separate X- and Y-bearing spermatozoa from human semen was first reported by Ericsson and co-workers (1973). This technique, which has received a lot of press in recent years, is based on the ability of Y-chromosome bearing spermatozoa to out-distance slower moving X-bearing spermatozoa in a column of viscous protein (BSA). According to the theory behind this procedure, the sperm cells that have successfully migrated through the BSA gradients should result in a Y-rich sperm cell fraction, which is then used for artificial insemination.

In Ericsson's first report, 80% of the sperm cells which successfully migrated through the BSA layers carried Y chromosomes. Shortly thereafter, researchers indicated that they had been unable to repeat Ericsson's initial trials (Ross and co-workers, 1975; Evans and co-workers, 1975). Since then, however, other laboratories have reported positive results with human semen in separating Y-bearing sperm populations using BSA gradients (Soupart, 1975; Dmowski and co-workers, 1979; Beenink and Ericsson, 1982).

In 1984 Ericsson reported in the popular press (Time Magazine, August 27, 1984) that 146 women who became pregnant using this sexing method at clinics licensed by his Gametrics Company, 112 females gave birth to male offspring, for a 77% success rate. Gametrics Inc. now has licensed clinics established across the USA and in countries outside of North America.

The first attempts to separate semen of farm animals using a BSA gradient procedure was reported in cattle (Faust and co-workers, 1976).

In this study, bovine spermatozoa that migrated through the layered 6% BSA were observed to have a significant increase in the percent of motile spermatozoa over that of the control spermatozoa not exposed to BSA. Similar findings for BSA-treated sperm cells were reported for fresh and frozen dairy and beef semen (Illyes and co-workers, 1977, 1978). A slightly modified procedure has been reported for treating boar semen with BSA prior to artificial insemination (Dixon and co-workers, 1977, 1978). As had been observed for bovine spermatozoa, the percent motile spermatozoa increased significantly when the motility readings for BSA-treated sperm cells were compared with pre-treatment readings. Similar findings with sperm cell motility have been reported for BSA-treated stallion semen (Goodeaux and Kreider, 1978). Recently, Maxwell and co-workers (1984) reported that when ram semen was treated with BSA prior to freezing, there was an improvement in post-thaw survival rates.

Observations from the studies with farm animals indicate that the BSA filters out seminal debris, lowly motile sperm cells and sperm cells with abnormal morphology. Ericsson (1977) had observed similar findings with human semen some years earlier. If this is the case, BSA may serve as a biological filter allowing for the progressively motile sperm cells to migrate through the BSA layers.

Sex ratios following insemination with BSA-treated semen has been evaluated in preliminary studies with the pig (Dixon and Kreider, 1980) the horse (Goodeaux and co-workers, 1978) and the cow (Beal and co-workers), however, no significant changes in the sex ratio were detected. At a recent conference on sheep reproduction, I. G. White's group from Australia reported a trend towards male offspring born following

artificial inseminating 87 ewes with control and BSA-treated ram semen. From all these initial studies with farm animals, there does not appear to be enough information available to evaluate if significant changes in sex ratios had occurred.

In one study reporting a large number of cows inseminated with BSA-treated semen from Charolais bulls (Ericsson and co-workers, 1980), there was some indication of a ratio change towards male calves born, however, there was not a consistent increase in the proportion of male offspring born across various groups. Further study is needed in this area before final conclusions can be made on the BSA approach as a sex preselection method for farm animals.

Using a different approach to altering the sex ratio of offspring born, Zavos (1983) from the University of Kentucky reported a 16 to 25% shift in the sex ratio towards females when he administered antibody-treated semen to female rabbits at the time of artificial insemination. In this procedure, semen was collected from the males and then incubated with a H-Y antiserum. According to Zavos, the rabbit sperm cells have a H-Y protein antigen on the cell surface membrane of the gametes carrying the Y chromosome. During incubation the specific H-Y antiserum attaches to the H-Y antigens on the male-producing sperm cells (those carrying a Y chromosome) selectively immobilizing or killing them. This leaves the female-rich sperm cell population (those carrying an X chromosome) available for fertilization following insemination of the female.

The H-Y antiserum is produced in the laboratory using male spleen cells from the same species to be inseminated. The male spleen cells are rich in H-Y antigen, so when they are injected into the animal they

elicit an antibody response, resulting in H-Y antibody containing serum for use in subsequent artificial insemination studies.

Recent efforts by Dr. Zavos have been directed towards developing a mouse monoclonal system that will produce a purer form of the H-Y antibodies. In addition, he is presently expanding his preconception sex determination procedure to include mice and cattle. Efforts are now being made to alter the procedure so that the H-Y antibody only immobilizes the male-producing sperm cell rather than killing them. It is hoped that the immobilized male-producing sex cells could then be revived to be used for subsequent inseminations, possibly shifting the sex ratio in favor of the male.

The idea of preconception sex determination has a great deal of potential for the livestock industry and possibly in the future for humans. With this approach, the sex ratio of the offspring could be altered without having to collect and transfer selected embryos, as would be the case with H-Y antibody embryos sexing procedures. Further research is warranted in this area.

In Vitro Fertilization (IVF).

In vitro is a term often used for "biological activity occurring in a test tube." Consequently, in vitro fertilization would imply that an ovum (egg) or ova (eggs) of a female could be fertilized with male sperm cells in a test tube, rather than in the reproductive tract of the female.

In vitro fertilization has been attempted in mammals for nearly a century. A multitude of studies attempting to induce fertilization in laboratory animal species have been reported over the last 30 years.

Excellent historical reviews on the study of in vitro fertilization are available (Chang, 1968, Brackett, 1975; Whittingham, 1975 Gwatkin, 1975). The first reported successful in vitro fertilization was with rabbit ova (Chang, 1959). In this study, it was clearly demonstrated that rabbit spermatozoa were required to undergo capacitation (biochemical and structural changes) before achieving the capacity to penetrate the zona pellucida of the ovum.

Prior to 1975, in vitro fertilization of mammalian ova had been reported in more than 10 different species. When the criteria for successful in vitro fertilization in laboratory animals includes the birth of live offspring of both sexes after transfer to recipient females, the rabbit (Chang, 1959), the mouse (Whittingham, 1968) and the rat (Toyoda and Chang, 1974) were the only species prior to 1980 in which successful in vitro fertilization had been demonstrated. In other laboratory animal species pregnancies have been established, but live births did not result or were not reported.

Over the years, attempts with in vitro fertilization of ova from larger mammals has been much less successful than those with laboratory animals. Scientists by the mid-1970's concluded, as they had on numerous occasions in the past, that the production of live offspring from larger mammals and man via in vitro fertilization would likely not ever be accomplished unless a major breakthrough was discovered.

On July 25, 1978, Louise Joy Brown was born to Mr. and Mrs. J. Brown of England, the first human birth recorded in the world resulting from a human ovum fertilized in a test tube (Steptoe and Edwards, 1978). The embryo resulting from in vitro fertilization was transferred back into the uterus of the mother whose oviducts had been previously

removed. This birth was on the front page of the newspapers the world over. Steptoe and Edwards in their small laboratory in Cambridge, England did what others had been saying could not be accomplished. In fact, these scientists had to work secretly for years on this in vitro fertilization procedure to escape ridicule of the public and their peers. It is ironic that today these two scientists have been mentioned as possible recipients of the Nobel Prize for their important discovery.

At present, human in vitro fertilization (IVF) clinics are operating in many countries of the world, including the United States. Over 1,000 other IVF babies have been born to childless couples as a result of Steptoe and Edwards' procedure developed at the now famous Bourn Hall Clinic. IVF clinics are continuing to open in major cities throughout the world.

Attempts to produce viable offspring from farm animals with a complete in vitro fertilization procedure prior to 1980 were not successful. Some evidence of test tube fertilization and developing early-stage embryos had been reported in sheep, cattle and possibly the pig prior to 1980 (see Wright and Bondioli, 1981; Brackett, 1981). Although no live births had been reported from bovine oocytes in which fertilization had been attempted entirely in vitro, Newcomb and co-workers (1978) did report the birth of transplant calves from follicular oocytes that had been transferred to the oviducts of a previously inseminated recipient females.

Then on June 9, 1981, Ben Brackett and colleagues of the New Bolton Center for Animal Research at the University of Pennsylvania reported the birth of the world's first in vitro fertilized embryo transplant calf (Brackett and co-workers, 1981). Again, worldwide attention was

brought about by this birth. The calf, a healthy Holstein male named "Virgil", was conceived in a test tube, using the ovum and sperm of Holstein cow and bull. After several unsuccessful attempts to replicate the procedure that produced the first IVF calf, on Labor Day of 1983 Brackett's group reported the birth of four other in vitro fertilized transplant calves (including one set of twins), which had resulted from a slightly modified IVF procedure (Brackett and co-workers, 1984). During the last one and a half years, one IVF calf was born in Japan in 1985, IVF calves have been born in Canada (Sirard and co-workers, 1986; Lambert and co-workers, 1986) and most recently another IVF calf was reported born at the University of Wisconsin. Several other laboratories now reportedly have bovine IVF pregnancies underway.

The only live IVF offspring produced in the world from both sheep and swine were produced at the ARC Animal Research Unit in Cambridge, England (Cheng et al., 1986). Alterations in capacitation procedures and temperature control in the culture system proved to be important components of the IVF system for these two animals. Scientists had attempted in vitro fertilization in these two species, without success, for over 35 years. Researchers at the Animal Husbandry Institute of Japan have recently reported the birth of the first IVF goat offspring. No IVF offspring have been reported to date in the horse and only limited success with in vitro fertilization procedures has been reported in non-human primates. However, recently Clayton and Kuehl (1984) have reported the birth of the first baboon IVF baby.

In animals, oocytes for in vitro fertilization procedure are harvested either by surgical collection (oviduct or aspirated from follicles), laparoscopy or from abattoir materials. Often oocyte donors

are pretreated with gonadotropins to prepare the follicles for aspiration. For oocyte maturation to occur, the environment for in vitro fertilization must mimic that of the reproductive tract of the female. The culture medium used in an IVF procedure must supply the necessary nutrients and factors needed for fertilization and subsequent mitotic cell divisions to occur. One of the major technical barriers to in vitro fertilization in animals has been preparing spermatozoa for ovum penetration (spermatozoa capacitation and acrosome reaction). Also, oocyte maturation in vitro and developing a culture system for early-stage embryos add further difficulties to producing IVF offspring at the present time.

In vitro fertilization in farm animals could be used to maximize utilization of semen from a valuable male and the ova from a valuable cow. For example, one unit of frozen-thawed bull semen is used to artificially inseminate one cow, with the expectation of producing a single calf. Due to freezing loss and normal wastage of sperm cells in the reproductive tract of the cow, usually greater than 15 million sperm cells are packaged in each unit of frozen semen. With in vitro fertilization, fewer sperm cells from the same unit of semen would be needed to produce an embryo. Therefore, one unit of semen could be placed in a test tube with many ova from a valuable female, producing many embryos. These embryos could then be transferred to surrogate females, which would give birth to the full sibs all at the same time, yielding a more uniform offspring for the producer.

There are already a number of "world class" bulls that have died, leaving only a limited number of frozen semen units in storage. Often holders of stored frozen semen units from these sires consider then

priceless, and they are usually not for sale. In recent years, other holders of rare frozen semen have marketed it for \$5,000 or more per unit, and all that were available were purchased. With in vitro fertilization, many more embryos could be produced from each unit of this rare semen. This may have important implications for saving endangered species and aid in the fight against male infertility.

Since in vitro fertilization occurs in the test tube, there would be no need to have either the semen donor or the ova donor present to produce these embryos. With frozen semen and frozen ova stored in liquid nitrogen, the livestock producer could produce embryos for a seedstock herd from bulls and cows that have been dead for 20 years or more. Having this potential, a livestock owner would have the unique opportunity to direct the genetic input into his herd even though the original seedstock is no longer available to him. A new bloodline developed in his herd could be crossed with germ plasm of his foundation stock from a decade past.

It is not out of the realm of possibility that a producer could select various male and female genetic combinations from a catalogue and then have the ova fertilized for him at a commercial IVF center. The embryos could be sent to him by air and he would place them into his herd of surrogate cattle to produce the desired calf crop, in whatever season the producer wished. The national and international marketing potential of this approach to embryo production for the livestock industry would be almost impossible to fathom.

Fertilization of the Ovum by Sperm Cell Microinjection.

Studies at several research groups in the USA and abroad have been attempting to develop techniques to develop offspring from microinjection of sperm cells into unfertilized ova (Uehara and Yanagimachi, 1976; Thadani, 1980; Markert, 1983; Shaikh and co-workers; 1984 and others). The possibility now exists that sperm cell microinjection techniques can be developed for cattle (Iritani and co-workers, 1984; Westhusin and co-workers, 1984). At present, the basis of this approach to sperm microinjection would be to either fertilize ova with the smallest amount of rare semen, or to produce an offspring from one male (two sperm cells) or two males (one sperm cell from each male) without the incorporation of female genetic material.

Using the first approach for rare semen, the ovum would be activated by a single sperm cell, either in the female or in vitro, after which the activated ovum would be subjected to sperm microinjection to introduce a second sperm cell into the ovum. Once this has been completed, the genetic material from the first male's sperm cell (male pronucleus) would be removed from the ovum with a microsuction needle, leaving the genetic material from the female and the sperm cell of the second male to join together (syngamy) to form a 1-cell embryo.

With the second approach, two sperm cells are injected into an ovum and the female genetic material (female pronucleus) would then be removed with a microsuction needle, leaving the genetic material of the two sperm cells to form a developing embryo. With the latter approach, generally 2/3 of the offspring born would be males and 1/3 females. The possibilities of producing an offspring from two bulls or two sperm

cells from one bull (and no female genome) could revolutionize the bull stud industry in the future.

The possibility exists that freeze dried sperm cells could be used to fertilize farm animal oocytes with this technique in the future. It is interesting to note that at least two calves have been born following oocyte fertilization with previously dried bovine sperm cells (Meryman, 1960; Graham and co-workers, 1974).

Freezing Mammalian Embryos.

In 1972, David Whittingham (England) gained international attention when he produced the first mammalian offspring (mice) from embryos that had been frozen at -196°C in liquid nitrogen (LN_2). The next year, Ian Wilmut reported the birth of the first calf born in the world (June) from a frozen embryo at the Animal Physiology Research Unit in Cambridge, England (Wilmut and Rowson, 1973). This offspring, "Frosty II" (a healthy bull calf), had been frozen as an embryo for 6 days in liquid nitrogen before being thawed and transferred to a crossbred recipient cow. Following this event, researchers all over the world began working diligently on the development of an applied procedure to be used by the cattle industry.

In 1978, the first frozen-thawed embryo transplant calf was born in the USA at Carnation Genetics (California). During 1980-81, it was estimated that over 100 frozen embryo calves were born on cattlemen's ranches in the United States. A five-fold increase in the number of frozen embryo calves born was estimated for 1982, and in 1983 layman publications began carrying advertisements of both small and large commercial companies offering this service to the cattle industry. One

estimate indicated that 21,000 frozen-thawed embryo transplant calves were born in North America in 1984 and double that number were expected for 1985. Although scientists still consider this technology to be in the developmental stages, it would appear that the future for freezing farm animal embryos is indeed promising.

Successful pregnancies have been reported in other laboratory animals (Table 5), including the rabbit (Bank and Maurer, 1974), and the rat (Whittingham, 1975). The first frozen-thawed transplant lambs were produced in England during the mid-1970's (Willadsen and co-worker, 1974) and in 1982 the first reported frozen embryo lambs were born in the USA (Louisiana) (Sims and co-workers, 1982). Today, several commercial transplant units in the United States have incorporated this technology into their services for sheep producers. Also, live offspring were first produced from frozen-thawed goat embryos (Bilton and Moore, 1976) and it was not until 1986 that the first frozen goat embryo offspring were produced in the United States (Pendleton and co-workers, 1986). In 1982 the first frozen-thawed embryo foal was born to a recipient mare in Japan (Oguri and co-workers, 1982) and in 1983, Colorado State University reported the birth of the first frozen embryo transplant foal in the North America (Takeda and co-workers, 1984a). The first frozen embryo transplant human baby was produced by Alan Trounson's group in Australia in 1984.

Although 30 years of effort was made by researchers, it was not until recently (August, 1984) that live offspring produced from frozen-thawed swine embryos was reported to have occurred at Trans World Genetics in California (Genetic Engineering News, September, 1984). At this

TABLE 5.

Successful Pregnancies have been Reported
with Frozen-Thawed Mammalian Embryos

<u>Species</u>	<u>Researchers</u>
Mouse	Whittingham <u>et al.</u> (1972)
Cow	Wilmut & Rowson (1973)
Rabbit	Bank & Maurer (1974)
Rat	Whittingham (1975)
Sheep	Willadsen <u>et al.</u> (1976)
Goat	Moore & Bilton (1976)
Horse	Oguri <u>et al.</u> (1982)
Pig(?)	Allen <u>et al.</u> (1984)
Man	Trounson & Wood (1984)

writing, the frozen porcine embryo breakthrough has not yet been reported in the scientific literature.

The potential applications of freezing farm animal embryos have only recently been realized by the livestock industry. For example, transferring frozen-thawed embryos at the time of choice will dictate the season of the year that offspring would be born. Storing frozen embryos would allow producers to sell outstanding females at production sales, or even to market the entire herd at peak prices. Freezing embryos would allow for disease testing while embryos are kept in quarantine or saving valuable germ plasm by collecting and freezing embryos from quarantined herds destined for slaughter (e.g. Brucellosis). Embryo freezing is beneficial when valuable donor females produce more good quality embryos than the number of recipients available on the day of transfer. Without freezing, these embryos are often times discarded or in desperation placed in recipient females that have virtually no chance for a successful pregnancy. The latter is often the case with human IVF procedures.

What are the success rates from frozen embryos? In cattle, for example, the pregnancy rates following non-surgical transfer of frozen-thawed bovine embryos usually range between 30 to 55%. Some of the more experienced professionals have reported pregnancy rates approaching 70%; however, this is often mastered using a surgical paralumbar approach to place the embryo into the uterine horn of recipient females. Scientists generally are optimistic that average success rates for transferred frozen-thawed bovine embryos will improve with additional basic research on the cryobiology of mammalian embryos.

Frozen-thawed embryo transfers in sheep, goats and horses have been completed primarily in research studies, with some commercially transferred. However, too few transfers have been made to give a meaningful estimate of the average pregnancy rates following transfer in these species.

Recent advances have been made that have refined various aspects of this freezing technology. A new freezing unit (machine) has been developed in Louisiana that freezes embryos without the use of liquid nitrogen (Voelkel and co-workers, 1984a; Viker and co-workers, 1984a). This freezing unit uses a standard 110-volt electrical power source, and small thermoelectric cells in the cooling chamber reduce the temperature of the embryos at a controlled rate. Laboratory evaluation of this new freezing method shows similar results to that of the standard LN₂ vapor freezing units now on the commercial market. Different embryo cryoprotective agents, such as glycerol and sucrose, are now being used in place of dimethylsulfoxide (DMSO) for freezing and thawing of mammalian embryos. Vital stains for embryos, such as fluorescein diacetate (FDA), are now being used to assess the viability of embryos prior to and following freezing (Looney and co-workers, 1982a, 1982b). These stains assist in detecting the highest quality (viable) embryos for transfer to recipients. Vital staining techniques have improved pregnancy rate percentage for those frozen-thawed embryos transplanted in cattle.

A new culture system has been developed for use in holding embryos prior to and following freezing. Embryos are placed in a special medium and then allowed to develop in culture dishes on a layer of cells (monolayer). These cell cultures were derived from special cells (fibroblasts) taken from bovine uterine tissues at slaughter. Using this

monolayer culture system, cattle embryos develop outside the female for 1 to 4 days with excellent viability (Voelkel and co-workers, 1984b). In vitro culturing allows for careful evaluation of embryos of questionable quality before selection is made for transfer.

Stanley Leibo from Rio Vista Genetics (Texas) has recently developed a method of freezing embryos in semen straws (units) with both the cryoprotectant and the rehydrating agent stored in the same unit when the embryo is frozen (Leibo, 1982, 1983, 1984; Leibo and co-workers, 1982a, 1982b). With this procedure (termed the "One-Step" method), embryos can be removed from LN₂ storage, warmed and placed directly into the uterus of the recipient cow with a Cassou AI catheter, similar to the methods used by producers that artificially inseminate their cattle with frozen-thawed bull semen.

Individual cattle embryos in a small amount of a cryoprotectant-buffer medium (e.g. glycerol and Dulbecco's PBS) are placed in .25 or .50 ml plastic semen straws, and the remainder of each straw is then filled with a specific concentration sucrose-buffer solution. The cryoprotectant-buffer medium containing the embryo is separated from the sucrose-buffer solution by a small air bubble (Figure 4). The embryo is maintained in position within the straw by a second air bubble.

Following freezing, the straws are stored in a liquid nitrogen tank (-196°C) until the time that suitable recipient females are available for transfer. Straws containing the embryos are then either thawed in warm water (e.g. 37°C) for approximately one minute or allowed to thaw at room temperature in air. Each straw is then shaken briskly to move the thawed embryo from the cryoprotectant-buffer medium into the diluted sucrose-buffer solution within the straw. The shaking movement

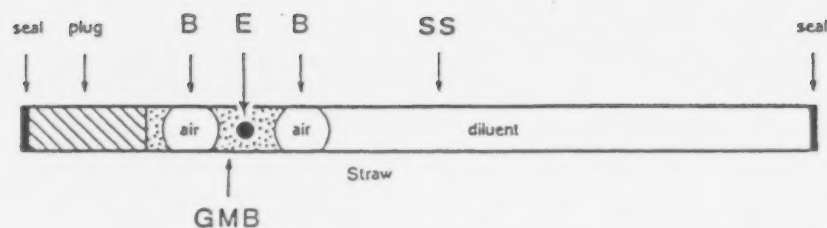


Figure 4. A diagram of a "One-Step" .25 milliliter straw containing the glycerol-buffer medium (GBM), an embryo (E) and the sucrose solution (SS). Note the two air bubbles (B) used to separate the two solutions until the straw is briskly shaken to move the embryo into the sucrose solution prior to transfer (Leibo, 1984).

dislodges the bubbles allowing the two solutions to mix. The sucrose-buffer solution aids in rehydrating the embryo after thawing and the warmth of the uterus brings the thawed embryo up to body temperature of the cow.

LSU's first transplant calf born from this new embryo handling procedure, a Hereford x Angus crossbred male weighing 115 pounds, was delivered at term by a Maine Anjou crossbred recipient on January 25, 1983. It has now been estimated that well over 375 transplant calves resulting from this procedure have been born in the United States. Presently, several modifications of this embryo handling method are now under evaluation.

This One-Step procedure alleviates the need for micropipettes, sterile dishes, a stereomicroscope and individually handling of individual embryos before their transfer to recipient females. Using method, there are no intermediate steps of opening the packaged unit and handling the embryos, as has been the case in the past. The thawing and transfer procedure is relatively simple, taking only a few minutes to complete.

This packaging method will likely be the key to development of cattle frozen embryo banks in the future. With the one-step packaging, frozen embryos of choice could be obtained from an embryo bank and transplant them directly into recipients at a time of convenience. This could be done in place of artificially inseminating these females with frozen-thawed semen. A top quality replacement herd could be established in a reasonable length of time using this new embryo technology. Several embryo banks are already in existence for cattle embryos and at

least one bank is now in existence for human embryos in Monash, Australia.

How long might mammalian embryos remain viable stored in liquid nitrogen? This question has yet to be answered. It has been reported that processed bull semen frozen in liquid nitrogen for 30 or more years has produced live viable calves when placed in the reproductive tract of suitable females.

Studies in England have been conducted to evaluate the viability of mouse embryos frozen in liquid nitrogen for over 10 years. Results showed that long-term storage of mouse embryos did not significantly reduce their viability when transferred to recipient mice. It has been reported that bovine embryos frozen for 6 or 8 years in liquid nitrogen have produced viable transplant calves. At this time, there is no reason to believe that frozen bovine embryos will be any less viable in long-term storage than that of frozen bull semen. Since the first frozen-thawed embryo transplant calf was only produced in 1973, it will be a matter of time before this question is fully answered. Since only a small number of frozen-thawed human embryos have been born in the world to date, this question can not be answered for human embryos at this time. Most researchers are optimistic that long-term storage will not alter the viability frozen human embryos.

Frozen Storage of Oocytes.

There has been considerable experimentation on freezing mouse and cattle embryos over the last decade. The freezing of mouse embryos has now become the standard to test new cryoprotectants and cooling procedures for other mammalian species. The methodology for freezing

bovine embryos has now become standard enough to be used across the majority of commercial embryo transplant companies presently in operation. However, relatively little research has been reported on the success of freezing ova of laboratory and farm animal species. There is no evidence to date that live transplant offspring have been produced from a previously frozen oocyte in animals. Since in vitro fertilization procedures are in place for human oocytes, it is logical that this area of research should become active during the next 3 to 5 years. Correspondingly, in 1986 a human baby was reported born that had resulted from the transfer of a frozen-thawed human oocyte (Ref.).

Leibo (1980) has reported that mouse ova have been calculated to have a different hydraulic conductive range than do later-stage mouse embryos. There also appears to be some difference between the two stages when temperature coefficient of water permeability are calculated for various mammals. According to Leibo, mammalian ova have a relatively high coefficient of water permeability which means that a small reduction in temperature greatly decreases the rate that the ovum can lose water. For ova, survival post-thaw depends critically on the cooling rate during the first process.

Preliminary studies on oocyte freezing at this laboratory suggest that the zona pellucida may be more susceptible to structural damage during freezing, resulting in a greater number of bovine ova breaking open at post-thaw than would be expected from freezing day 6 or day 8 cattle embryos. The physical and chemical properties of the acellular bovine zona pellucida have recently been shown to be different when oocytes were compared to morulae and early-stage blastocysts (McFarland, personal communications).

A primary reason for not being able to produce offspring from frozen-thawed mammalian ova is that a proficient in vitro fertilization procedure is needed to generate the embryos for transfer to recipient females. Only recently have IVF procedures produced live offspring in cattle, sheep, goats and swine. The human is the one species that has in place efficient IVF methodology, which usually results 50 to 85% fertilization rate for normal oocytes (Steptoe and Edwards, personal communication, 1982). This obviously is an important area of research in farm animals and humans.

One of the areas presently under study at LSU is the potential of collecting viable oocytes from females following their death. These oocytes would then be frozen and stored until they are placed in in vitro fertilization to produce embryos. A dairy or beef cow rarely produces more than eight or ten live calves in their productive lifetime. Research has shown that these females contain literally thousands of potential oocytes in their ovaries that are never ovulated, therefore, wasted as far as producing offspring (see Table 4). The idea would be to allow the female to produce as many offspring as she is capable of in her natural productive life.

When physical condition of a female dictates her removal from the herd, sell the female and excise her ovaries at slaughter. The ovaries are packed in ice and sent to a commercial IVF center where the follicular oocytes are extracted from each ovary with a needle and syringe. The remaining oocytes are collected from minced ovarian tissue. More ova could be removed from each ovary than calves ever produced in the female's lifetime. As the animal's natural born offspring prove themselves genetically, her frozen ova (collected after her death) could

then be available for in vitro fertilization as desired. Developing this procedure of ova collection for cattle and other farm animals could be used in the case where a viable, healthy female was killed by accident (e.g. lightning, drowning) or died from an illness at an early age, without contributing many offspring in the herd.

The Potential for Frozen Storage of Spermatozoa

Older textbooks indicate that an Italian, Lazzaro Spallanzani, was first to document the use of artificial insemination (AI) in domestic canine (spaniel bitch) in the early 1780's. This report came over 100 years after Ham and Leeuwenhoek microscopically identified the sperm cell and postulated as to its natural function. Prior to the report by Spallanzani (translated in 1782), legend has previously indicated that Arab horsemen routinely collected semen from the vaginae of covered mares and inseminated other estrual mares as early as the 14th century.

Following years of effort, Dr. John Hunter produced the first human pregnancy using artificial insemination in 1799 (reviewed by Rice and co-workers, 1957). In the late 1800's, the Russians began developing AI techniques for use in farm animals, and by the turn of the century AI was being used on government horse breeding farms. In 1914, Professor Anantea from Italy devised the use of the first artificial vagina for collecting canine semen and shortly thereafter the Russians developed artificial vaginae for rams, stallions and bulls (reviewed by Perry, 1968). The use of the artificial vagina for semen collection from farm animals is now considered to have been a very important step in making it possible for world-wide use of artificial insemination (AI) on farms and ranches.

In Europe in the early 1900's, practitioners began artificially inseminating dogs and horses on an experimental basis. During the 1930's, AI in cattle with fresh semen was being promoted for use by the cattle producers. Today, AI in cattle has developed into a multimillion dollar business in North America.

In a short paper, Polge and co-workers (1949) reported the first evidence of success in freezing and subsequent thawing of animal spermatozoa. This initial study is now considered to be a classical work since it describes the unique ability of glycerol to protect the male gamete and freezing and thawing process. The first calf was produced from frozen semen several years later (Polge and Rowson, 1952). The discovery of glycerol as a cryoprotectant for freezing spermatozoa has probably contributed more to the advancement of applied reproductive physiology of farm animals than any other finding to date.

The early use of glycerol and egg yolk in successful methods for freezing spermatozoa were initially reported for the ram (Emmens and Blackshaw, 1955), caprine buck (Frazer, 1962), stallion (Buell, 1963), boar (Hess and co-workers, 1957), bull (Polge and Rowson, 1952; Emmens and Blackshaw, 1955), buffalo (Roy and co-workers, 1956), domestic dog (Martin, 1963) and man (Sherman and Bunge, 1953; Sherman, 1964). The successful use of glycerol as a cryoprotectant for early sperm cell freezing studies with the domestic chicken (Brown and co-workers, 1963), turkey (Macpherson and co-workers, 1969) and fish (Blaxter, 1953) have also been reported.

During the 1960's and early 1970's there have been a multitude of reports on successful artificial insemination of sheep with frozen semen. Today, AI with frozen semen has become an integral part of sheep

breeding managements systems in Europe, New Zealand and Australia. Correspondingly, ram semen, as with bull and human semen, has been shown to remain viable following storage in liquid nitrogen for extended periods of time (Salamon, 1980). At present, many millions of cattle and pigs are artificially inseminated throughout the world every year, the majority of cattle with frozen semen and the pigs most often inseminated with fresh or chilled semen.

Freezing porcine spermatozoa was not developed for commercial use until a practical method was reported in the early 1970's by Ed Grahman's laboratory at the University of Minnesota. Although commercial boar studs have frozen porcine semen available for sale, swine producers in North America have been slow in accepting in their breeding programs. Frozen stallion semen has been in the past promoted as a commercial source of new genetics, however, most of the larger breed association still not allow for free use of artificial insemination procedures (without restrictions) for registry of foals. For the range in pregnancy rates expected from AI with frozen semen see Table 6.

The most successful sperm cell freezing procedure has been with bovine semen. The procedure is usually carried out in two steps at commercial bull studs. The first step includes a predilution warming of the semen in a water bath and the addition of an egg yolk buffer. The egg yolk provides lecithin and lipoproteins to protect the sperm cells during the cooling process, likely by preventing changes in the cell membrane permeability when temperatures are reduced. When the semen and egg yolk buffer has been mixed and cooled down to 5°C the addition of glycerol is added in the second step.

Table 6. Expected Fertilization Rates at Natural Mating and Pregnancy Rates from Frozen Semen for Farm Animals and Man^a

Mammal	Fertilization Rates from Natural Mating (Range)	Pregnancy Rates from Frozen Semen (Range)
Cattle	82-100%	45-75%
Sheep	80-93%	30-57%
Goats	85-96%	35-55%
Horses	55-90%	30-68%
Swine	95-100%	40-65%
Humans	25-50%	20-68%

^aData collected from multiple sources.

The added glycerol binds water and decreases the freezing point of the solution. Glycerol partially dehydrates the spermatozoa further reducing the selective freezing of water. It has been shown that damage results from the selective freezing of free water (ice crystals) both inside and outside of the sperm cell. This results in the concentration of other cell constituents and solutes outside the cell, thus changing the permeability of the cell membrane, altering vital cellular components inside the cell and causing leakage of solutes from the sperm cell. With the addition of glycerol less ice is formed in the mixture at any temperature. Since glycerol is generally considered toxic to bovine spermatozoa at 35°C, it is added with the buffer at a cool temperature.

Once equilibration of the sperm cells and glycerol has been completed (1 to 18 hours) semen is packaged at desired concentrations in either 0.5 or 1 ml glass ampules or the more popular .25, .33 or .50 ml capacity plastic semen straws for freezing. The standard vapor method for freezing bovine spermatozoa calls for a single layer of straws placed 5.5 to 6 cm above the liquid nitrogen level of a large storage unit. Once frozen the straws are placed in the liquid nitrogen (-196°C) for storage.

Since many bovine spermatozoa do not survive freezing, even with the best known methods, 15 to 30 million motile sperm cells are usually packaged per unit of semen to be frozen. This depends on the freeze-thaw survival rate of the male being processed. This freezing loss is over 50% with some male animals. Some bulls have semen that has excellent post-thaw motility while others have sperm cells that simply do not survive freezing. The reason for this can not be fully explained. It

is generally accepted that 10 million motile spermatozoa post-thaw is considered an acceptable number of cells optimal for AI in cattle. Research has shown, however, that 5 up to 10 million progressively motile sperm cells can be used per insemination without reducing pregnancy rates in cattle (Sullivan and Elliott, 1968; Hafs, 1971). In the United States, it is now estimated that over 70% of the 9 to 10 million dairy cows and a little less than 4% of the 36 to 37 million beef cows are AI with frozen semen. Since frozen semen in plastic straws can be stored more efficiently than glass ampules and the fertility of frozen spermatozoa packaged in plastic straws is equal to or greater than sperm cells packaged in glass ampules (Macpherson and King, 1966 and others), the plastic straw is now used world-wide in commercial semen banking for cattle.

The potential for male gamete production for AI is enormous. In the bull, for example, a healthy mature male can produce almost 10 billion sperm cells in a single day. Under routine semen extension and packaging of 20 million spermatozoa per semen unit for freezing, a bull could produce enough semen for over 180,000 inseminations per year. If the male was reproductively active for 8 years in a bull stud, he could produce enough semen for over 1.4 million seminations. This is dramatic when compared to the reproductive life of an average producing cow in the breeding herd. The cow has a 21-day estrous cycle which calculates; if not mated or inseminated, to the production of 17 ova for fertilization per year. Considering an average postpartum interval to pregnancy and a pregnancy of 283 days she would ovulate three to four ova per year. If this female had an 8-year reproductive life in the herd, she would only produce 24 to 32 ova in her productive life time.

The potential for developing freezing technology for species other than farm animals and humans is great. The need for this technology is especially important to help save endangered species and to preserve rare bloodlines in minor breeds and zoo animals (see Rowlands, 1964; Blakewood and Godke, 1986). Some success has been reported using fresh semen inseminations with various captive zoo animals, however, only a limited number of success have been documented using frozen semen to produce live offspring in endangered species (Seager and Platz, 1976). Unfortunately, many of these results remain unpublished.

In non-human primates the use of frozen semen would offer new breeding opportunities for captive population both at zoos and at research institutions. Sadler (1966) reported the first success in freezing semen from non-human primates. In this study, some post-thaw motility was observed in chimpanzee semen after 1 to 3 days storage at -196°C . The following year, Roussel and Austin (1967) reported better results with post-thaw motility of 22 to 30% for semen of the Rhesus monkey and 25 to 30% for chimpanzee semen after being stored in liquid nitrogen for 3 days.

Shortly thereafter, Kraemer and Vera Cruz (1969) first reported successful freezing and thawing of baboon semen, but the post-thaw motility was low. Three pregnancies have resulted from inseminating macaque monkeys with frozen semen in two different laboratories (Leverage and co-workers, 1972; Cho and co-workers, 1974), but the females aborted prior to term. The potential and the technology for freezing non-human primate semen seems to be evident, however, producing live offspring from artificial insemination has been a problem. Social behavior patterns, difficulty in handling with these animals for semen

collection and poor semen quality are also barriers that have been the most difficult to overcome.

The first pregnancy in women inseminated with frozen semen was announced to the public over 30 years. By 1964, 24 or more children were born as the result of insemination with frozen-thawed semen in the United States and Japan (see Sherman, 1963, 1964). In the late 1960's successful artificial insemination results with fresh and frozen semen were being reported from hospitals world-wide.

In one study, Langer and co-workers (1969) reported that following single or multiple inseminations of 158 women during one time interval, 156 conceived and 142 healthy AI children were born. By 1973, it was reported that nearly 600 births had been recorded from artificial insemination with low temperature stored human sperm cells (Sherman, 1973). In 1984 it was estimated that more than 250,000 babies had been born in the USA as a result of frozen-thawed human semen. One estimate was that 80,000 AI children alone were born in the United States in 1980.

One of the largest human sperm bank organizations in the United States (Georgia) now claims that it is sending out up to 100,000 inseminating units of human semen annually, and is expecting the market to increase in the future (The Observer, p. 3, February 7, 1983). Banking organizations pay between \$20 to \$40 per sample in most cases. The selling price per unit of semen usually starts at \$100 and goes upward. The success rate from frozen-thawed human semen now ranges from 20 to 68% (see Table 6). The pregnancy rates relate closely to the quality of semen processed and the banking organization freezing the semen. There apparently is a need for quality control in packaging of

human semen much as has developed with commercial bull studs in North America.

The development of modern cryobiology was accelerated in 1949, when Polge and co-workers showed that the introduction of glycerol as a freeze-thaw protective agent facilitated the preservation of spermatozoa during freezing. The preservation of human spermatozoa was neglected until the early 1950's (Sherman and Bunge, 1953). Early human trials used a slow freezing curve and storage in a solid carbon dioxide (-79°C). Bunge and co-workers (1954), using this approach, reported post-thaw survival rates of over 60% motility after 90 days of cold storage. Pregnancies did develop using this frozen-thawed semen under clinical conditions.

Today the basic approach to freezing human semen involves deluting the whole semen with either glycerol or a glycerol-egg yolk-citrate semen extender and then vapor freezing it, either rapidly or slowly over a period of 30 minutes to an hour. The extended semen unit is then plunged directly into liquid nitrogen for storage (-196°C). Using this basic approach, the post-thaw survival rates have been reported to range from 30 to 70% (Silbert, 1972; Beck and Silverstein, 1975; Friberg and Genzell, 1973; Sherman, 1973; Tyler, 1973 and others).

Glaub and co-workers (1976) made an effort to improve the quality of the ejaculate prior to freezing by filtering the sperm cells over bovine serum albumin (BSA). This technique had been reported early by Ericsson and co-workers (1973). The separated fraction was placed with semen extender (1:1 volume of egg yolk-glycerol-glucose-citrate medium) and cooled at a constant rate (12.9°C per minute) down to -80°C to -100°C . Results showed that the post-thaw motility was substantial

higher for the BSA-separated sperm cells than for the untreated semen. It was concluded that the BSA separation improved the post-thaw motility and may have a place in the improvement of human semen freezing procedures. The use of BSA as a biological activation and filter for farm animal spermatozoa has also been reported (see section on The Potential for Sexing Sperm Cells).

How long might mammalian sperm cells remain viable stored in liquid nitrogen? It has been reported that processed bull semen frozen in liquid nitrogen for 30 or more years has produced live viable calves when placed in the reproductive tract of suitable females. Recently, a healthy Holstein bull calf born in Wisconsin from frozen bull semen that was collected and processed at American Breeders Service (ABS) on November 19, 1953 (Advanced Animal Breeder, August, 1984). This is interesting in light of the first calf produced in this country from frozen-thawed bull semen was on May 29, 1953 in a Janesville, Wisconsin herd.

In Vitro Culture Systems for Embryos

Once fertilization has occurred in vitro, the pregnancy rate following transfer to recipient females is quite low for most animals studied. For example with the cow, ova fertilized outside the body must undergo development to a morphological stage equivalent to that occurring at 6 to 8 days post-fertilization for optimal transfer pregnancy rates to occur. To reach this stage of development post-fertilization an intermediate in vivo or an in vitro culture system is needed for the developing embryos to survive.

With an in vivo system, early or late stage embryos (e.g. cow) are surgically transferred to the oviducts of an intermediate host (e.g. rabbit) for a short period of time or until the embryos reach a late morula or blastocyst stage of development. The embryos are then recovered from the ligated oviducts and transferred to suitable recipient females.

In earlier studies, the female rabbit was the most often used intermediate host for short-term culture or for transport of mammalian embryos (Averill and co-workers, 1955; Hunter and co-workers, 1962; Hafez and co-workers 1963; Adams and co-workers, 1968; Rowson and co-workers, 1972 and others). Today, the most popular intermediate host animal for embryo engineering research would likely be the diestrous or anestrous ewe. Willadsen (1979) first described the use of an agar embedding procedure for micromanipulated embryos, which used the sheep as an intermediate host for in vivo culture. Subsequent studies by Willadsen and others laboratories have used the agar embedding procedure and the ligated oviduct of the ewe for in vivo culture of cattle, swine, goat, sheep and horse embryos prior to their transfer to respective recipient animals.

Although a multitude of embryo culture media have been tested for in vitro culture of mammalian embryos over the years, only a few remain for modern day culture of laboratory and farm animal embryos (see review by Wright and Bondioli, 1981). From recent studies with laboratory and farm animals, the most suitable type of medium for collection and short-term storage of embryos would be a physiological salt solution with a phosphate or Hepes buffer. This base medium is often supplemented with glucose, pyruvate, bovine serum albumin (BSA) or fetal bovine serum and

antibiotic-antimycotic. The basic recipe for the phosphate-buffered saline now used for various embryo media was originally described by Whittingham (1971) for mouse embryos, and its use has been reviewed for farm animal embryos by Betteridge (1977).

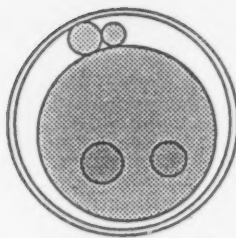
Reasonable survival rates (50%) have resulted from the transfer of later-stage bovine embryos previously incubated for up to 48 hours in phosphate-buffered medium supplemented with fetal bovine serum (Trounson and co-workers, 1976). However, the phosphate-buffered saline (PBS) medium is not able to maintain development of most laboratory and farm animal embryos from the 1-cell embryo through to blastocyst stage (Figure 5). Foote and Onuma (1970) reported that bovine embryos generally did not undergo more than one or two cleavage divisions in vitro, and rarely are more embryonic cell divisions recorded with various media. Only in the mouse (Whittingham, 1975) and the rabbit (Maurer, 1978) has complete pre-implantation development been achieved in vitro and that has been accomplished with a bicarbonate-buffered medium.

Most researchers now agree that any of the fluid medium in vitro culture systems now available will support viable later-stage embryo development for up to 48 hours, after which transfer pregnancy rates decline markedly. Most commercial embryo transfer companies allow harvested bovine embryos to cool to room temperature and then culture the embryos overnight only as the need arises. It is now clear that improved embryo culture procedures need to be developed for farm animal embryos in the future (Betteridge, 1977; Wright and Bondioli, 1981).

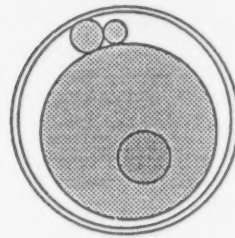
One of the embryo culture systems showing the greatest promise for both early-stage and later-stage mammalian embryos is the uterine fibroblast monolayer co-culture system. Salomon and Sherman (1975) indicate

Figure 5

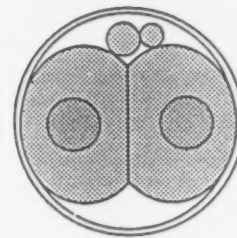
EMBRYO STAGES



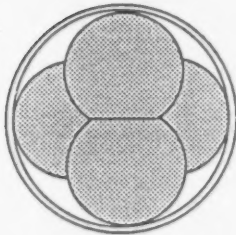
PRONUCLEAR



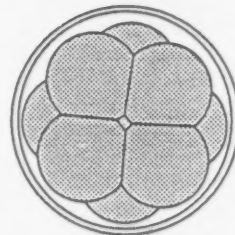
ONE-CELL



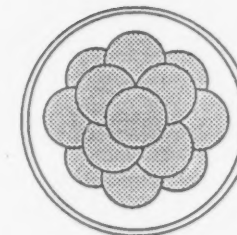
TWO-CELL



FOUR-CELL



EIGHT-CELL



SIXTEEN-CELL



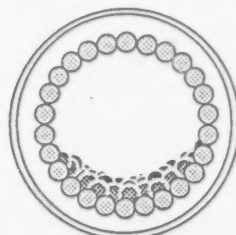
COMPACTION



MORULA



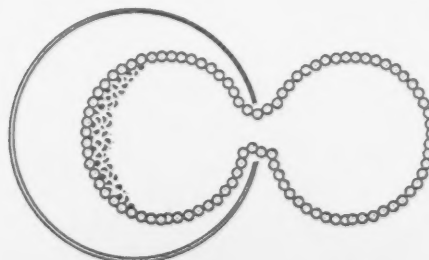
EARLY BLASTOCYST



BLASTOCYST



EXPANDED BLASTOCYST



HATCHING BLASTOCYST

that blastocysts, in the presence of an appropriate defined medium (supplemented with fetal bovine serum), would attach to the surface of sterile plastic or glass dishes and that embryonic trophoblastic cells would grow out of the embryo as a monolayer. This observation helped verify that the hatched blastocyst in utero is making an effort to come in close apposition with the uterine endometrial lining at a relatively early stage of development.

Mouse blastocysts were first shown to develop a characteristic outgrowth of trophoblastic cells when cocultured with a layer of live or irradiated HeLa cells (Cole and Paul, 1965). Also, good success has been reported when later-stage mouse embryos were co-cultured to hatching with liver cells, L-cells and those derived from a JLS-VII teratocarcinoma (Glass and co-workers, 1979). In the latter study there was no difference noted among the three cell types for in vitro culturing mouse embryos.

In the early 1980's, commercial embryo transplantation developed and expanded for farm animals. Shortly thereafter, these commercial transplant units began expressing an urgent need for a viable in vitro culture system. Subsequently, Kuzan and Wright (1982a) reported noteworthy results when a monolayer of bovine fibroblast cells of either uterine or testicular origin were used in a MEM co-culture system with morula-stage bovine embryos. With this in vitro system, 40% of the morulae developed into hatches blastocysts compared with only 3.4% for the control medium without the fibroblast cell monolayer. Kuzan and Wright (1982b) have shown that uterine fibroblasts also give good culture success with swine embryos when used in a co-culture in vitro system. These results confirmed the initial findings of Salomon and Sherman (1975), who reported

mouse blastocyst implantation in vitro when a monolayer of mouse uterine cells was used as a substratum.

In a more recent study, bovine embryos were bisected via micro-manipulation and the embryo halves (demi-embryos) were then used to compare Ham's F-10 culture medium alone with a co-culture system of Ham's F-10 medium and a monolayer of bovine endometrial fibroblasts (Voelkel and co-workers, 1985a). A diagrammatic scheme for preparing the uterine fibroblast monolayer system is shown in Figure 6. Results during the 72-hour culture period showed that the endometrial fibroblast monolayer co-culture system was superior over the Ham's F-10 control medium for culturing day 6.5 to day 7 bovine embryos. Some of the embryos that were left on the monolayer remained viable for 15 or more days in culture. Further studies in this laboratory have confirmed that the monolayer enhanced embryo viability over culture medium alone (Wiemer and co-workers, 1987). Recently, there has been interest developed for the use of the uterine fibroblast monolayer system to culture embryos of other mammalian species, including zoo animals and man.

Despite much effort to develop embryo culture media suitable for mammalian species, the culture success rate has been low. The fibroblast monolayer system seems to be a logical step for in vitro culture of both early- and later-stage mammalian embryos, however, further testing and evaluation are needed before final predications can be made in this area. One recent advancement now being evaluated for use in embryo culture and transplantation is the introduction of trophoblastic vesicles into the culture medium with the embryos prior to transfer. Preliminary reports suggest that trophoblastic vesicles (TRV) used in con-

PREPARATION OF BOVINE UTERINE FIBROBLASTS

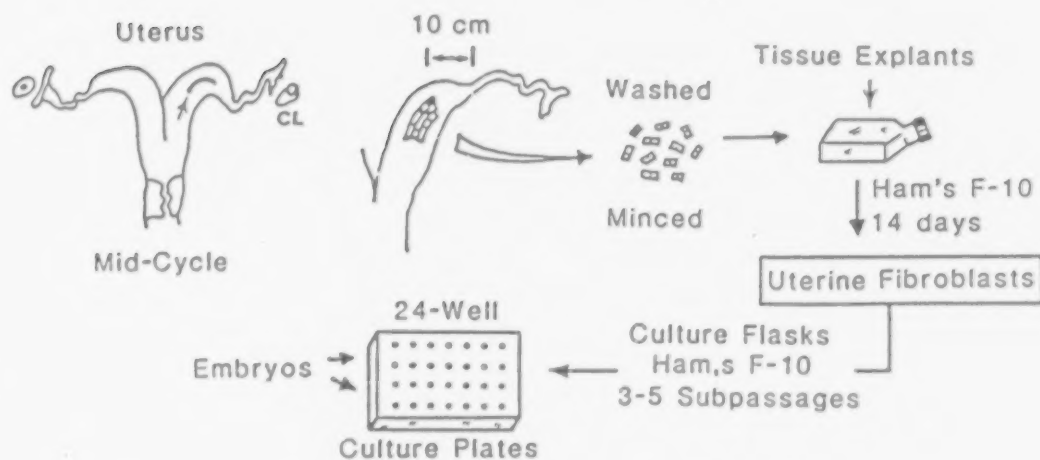


Figure 6. An outline of the scheme used in preparation of the bovine uterine fibroblast monolayer system for in vitro culture of later-stage bovine embryos (Voelkel *et al.*, 1985).

junction with bovine embryos may increase culture viability and possibility transfer pregnancy rates in recipient cattle.

Heyman and co-workers (1984) reported trophoblastic tissue fragments (TRV) from day-14 bovine embryos and day-11 to day-13 ovine embryos placed into the uterine horn (ipsilateral to the CL) of cyclic heifers and ewes during diestrous resulted in extended estrous cycle lengths. In this study, the placement of exogenous trophoblastic tissue into the uterus (day 12) of the cyclic female inhibited normal luteal regression, thus extending cycle length. Martal and co-workers (1979) had previously suggested that an antiluteolytic substance may actually be produced by the trophoblastic cells rather than from the embryonic disc cells, as was previously thought.

In a co-culture study with bovine embryos, Camous and co-workers (1984a,1984b) demonstrated that TRV placed in the culture medium of early-stage embryos (1- to 8-cell stages) enhanced embryo development in vitro (46% vs. 18%) when compared with those cultured in the same defined medium, but not co-cultured with TRV. A group of these embryos co-cultured with TRV were then transferred to recipient females, resulting in a 40% pregnancy rate. It was concluded that bovine TRV released one or more unknown substance(s) that promotes cleavage of early-stage bovine embryos.

This finding may be of great importance to our understanding of developmental embryology and to develop more efficient embryo culture systems. Bovine embryos, for example, have an 8- to 12-cell stage "block" that usually occurs during in vitro culture of early-stage embryos. The reason for the developmental block is not clear, but it certainly has hampered the development of an optimal early-stage embryo

culture system for years. Unlikely the other mammals evaluated, the human embryo has given no evidence that it has the developmental block during early embryo development (Edwards and co-workers, 1981).

Field studies are presently underway in France to evaluate the efficacy of transferring one or two bovine TRV with each frozen-thawed bovine embryo. If the transplanted TRV remain viable, they should inhibit luteolysis in the recipients, thereby potentially increasing the overall transfer pregnancy rate. It is interesting to note that Plante and co-workers (1985) have suggested the trophoblastic tissue may actually be luteotrophic in its action rather than antiluteolytic in nature as proposed by Heyman and co-workers (1984). Furthermore, if the TRV promotes cleavage, as in the early-stage embryo, this should also be an added benefit to embryo viability in culture and subsequent transfer pregnancy rates.

Additional studies with bovine trophoblastic cells in this laboratory indicates that TRV can survive freezing and storage in liquid nitrogen for in field use (Rorie and co-workers, 1987^a), but do not reform as TRV from a single cell suspension (Rorie and co-workers, 1987^b). Recently, Pool and co-workers (1987) have shown that bovine embryos (early morulae) can be successfully cultured in vitro after being inserted into the lumen of the TRV. Trophoblastic vesicles should not be overlooked as an aid in the culture of human embryos.

Sexing Mammalian Embryos

From both the research and the applied viewpoints, the future possibility of developing new procedures using embryo freezing and micro-manipulative techniques with mammalian embryos appear to be endless. In

addition, other new procedures and techniques, supplemental to micromanipulation, will likely be developed to aid embryo engineering research and enhance the commercial embryo transfer industry in the coming years. One possibility is to be able to sex embryos before their transfer to recipient females (Seidel, 1980a).

During the last 10 years most attempts at sexing bovine embryos have been with chromosome analyses (e.g. karyotyping) on cells extracted from the embryo proper (Hare and Betteridge, 1978; Moustafa and co-workers, 1978; Singh and Hare, 1980; Winterberger-Torres and Popescu, 1980). Chromosomal analysis is performed on cells in the metaphase stage of mitosis. In cattle and dogs for example, the X and Y chromosomes are morphologically different from each other and their autosomes. In the other farm animals, however, the X chromosomes are morphologically similar to the autosomes, so sex determination requires a full complement of chromosomes to evaluate a spread under the microscope (Hare and Betteridge, 1978).

These procedures are often time consuming, considered by most to be too tedious and require a well trained technician to give meaningful results. Although several research laboratories have had respectable results with karyotyping and transferring bovine embryos, most commercial transplant units that have used this approach to sexing embryos indicate that it lowers transplant pregnancy rates.

With increased interest in embryo freezing technology and micromanipulation, attempts are intensifying to make embryo sexing a simple and efficient procedure for use in commercial embryo transplant stations. Researchers have already reported success using the H-Y antibody tech-

nique for determining the sex of mouse pre-implantation embryos (White and co-workers, 1982, 1983; Wachtel, 1984 and others).

In an earlier review of the subject, Ohno (1979) indicated that a histocompatibility antigen (H-Y antigen) was present in or on the surface of male cells of those mammals evaluated, but that this antigen was not found closely associated with corresponding female cells. In rats, rabbits, guinea pigs, humans (Wachtel and co-workers, 1974), cattle (Ohno et al., 1976) and dogs (Hare and Betteridge, 1978), for example, the H-Y antigen is common. Eichwald and Silmer (1955) first recognized the presence of a histocompatibility antigen when inbred female mice would not accept male skin grafts from the same highly inbred strain of mice. Correspondingly, females would accept skin grafts from other females of the inbred strain. An antiserum to H-Y antigen can be produced within inbred strains of mice by inoculating females over time with male cells. With this in mind, researchers then developed specific antibodies to the male H-Y antigen (primarily of the IgM subclass) in hopes of devising a method of identifying male and female embryos immediately following their collection from the donor.

Most approaches now being tested use a fluorescent-labelled H-Y antibody so the sexing procedure can be completed while viewing the embryos under an inverted microscope with a fluorescence attachment. Progress in this area has been slower than expected due to the unexpected difficulty developing the H-Y antibodies. Expression of histocompatibility proteins on the cell surface of pre-implantation murine embryos has now been well documented using a H-Y antiserum (White and co-workers, 1982, 1983). These male-specific proteins have been detect-

ed in embryos from the 2-cell stage through the blastocyst stage of development.

In a preliminary report evaluating a small number of bovine embryos, Wachtel (1984) suggested that fluorescent H-Y antibody techniques may become a useful tool in bovine embryo sex determination in the future. White and co-workers (1986a) have now developed the noninvasive immunofluorescence assay for the detection of H-Y antigen with an overall accuracy of 85 to 90%.

With this H-Y antibody fluorescent detection procedure, embryos maintained in holding medium are subjected to H-Y antiserum and cultured for 45 minutes. Following washing, the embryos are placed in medium with FITC-labelled antiserum antibody (goat origin) for 45 minutes. The embryos are then washed three times with medium and evaluated under fluorescent microscopy. After evaluation, embryos of desired sex (fluorescing or non-fluorescing) are transferred to recipient females. The presence of H-Y antigen on the surface of sheep, swine and cattle pre-implantation embryo has now been documented using H-Y antiserum (White and co-workers, 1984, 1985, 1986b, 1986c, 1986d). Results thus far suggest that the noninvasive detection of H-Y antigen is currently the most promising to sexing mammalian embryos prior to transfer. The use of H-Y antiserum has also been evaluated for possible use in pre-conception sex determination using the rabbit as a model (Zavos, 1983). The findings from this study also appear to be encouraging certainly from the practical application viewpoint.

In a recent news release the Institut Nationale de la Recherche Agronomique (INRA) in France has announced that they have developed a DNA probe technique for use in sexing 7-day-old bovine embryos. Previ-

ous research efforts have given encouraging results using a DNA probe to sex mouse embryos. A similar approach using a specific DNA probe to sex bovine embryos is presently being evaluated by a commercial embryo transplant company in Texas. Results from the latter experiments have not been reported.

Today, there are continued claims of embryo sexing antibodies and/or embryo sexing kits for cattle being evaluated under field transplant conditions, however, these results have yet to appear in the scientific literature. Developing an efficient embryo sexing procedure for farm animals must be ranked as one of the highest priorities for future embryo engineering research.

Genetic Engineering — Its Potential for Farm Animals.

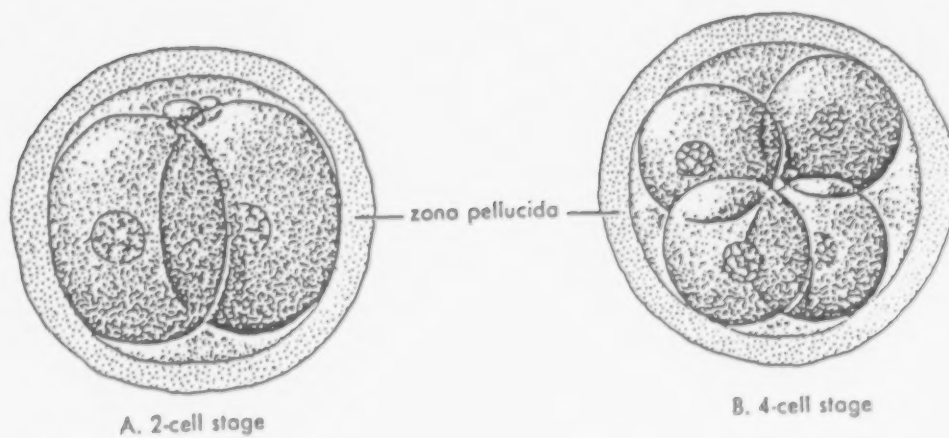
Genetic engineering has recently been redefined for animals as those technologies that induce direct changes in genetic materials (genes) or genetic frequencies (genotype) or that change life history characteristics of individuals or the genetic material (gametes) produced by that individual (see Rutledge and Seidel, 1983). In recent years the definition had to be redefined to include aspects of the new technology that were never thought before possible by the scientific community.

Micromanipulation of Embryos to Produce Multiple Offspring

The fertilized ovum of farm animals divides into two approximately equal size cells, called blastomeres, within hours after the sperm cell has entered the ovum (time of fertilization). The 2-cell embryo (Figure 7) then divides in a systematic pattern into 4 cells, 8 cells, 16 cells and so on until it reaches the 64-cell, 128-cell or greater stage of

Figure 7

Early-Stage Embryos



development. Shortly thereafter, a small cavity forms in the center of this spherical mass of cells called a blastocoele. Embryos at this stage of development (generally 7 to 9 days of age) are called blastocysts. As the blastocyst develops it has two characteristic cell types, the inner cell mass which subsequently forms the fetus and the trophectoderm which develops into the placenta (fetal membranes).

Micromanipulation of embryos to produce multiple offspring is a newly developed technique in farm animals, which involves opening the outer covering (zona pellucida) around the blastomeres with a finely drawn glass needle and surgically dissecting the cell mass of the embryo into individual cells or embryo parts. Since each blastomere at the early stage of development is capable, in itself, of developing into individual offspring, this approach has been employed to produce identical twin, triplet and quadruplet offspring from a single embryo in farm animals. One method for producing multiple offspring from a single early-stage embryo is illustrated in Figure 8.

In 1979, Steen Willadsen of Cambridge, England was accredited with a major breakthrough in farm animals when he produced the first live sets of identical twin offspring (lambs) from micromanipulation of early-stage sheep embryos (Willadsen, 1979). Two years later he reported the birth of identical twins and a set of triplet calves from micromanipulation of early-stage cattle embryos (Willadsen and Polge, 1981).

Willadsen used microsurgery techniques developed in mice on sheep and cattle embryos to produce genetically identical offspring. A key to the success of this procedure revolved around the use of the sheep as an intermediate host for the micromanipulated embryos. Early-stage embryos

BLASTOMERE TRANSFER

FROM 8-CELL TO 4-CELL EMBRYO

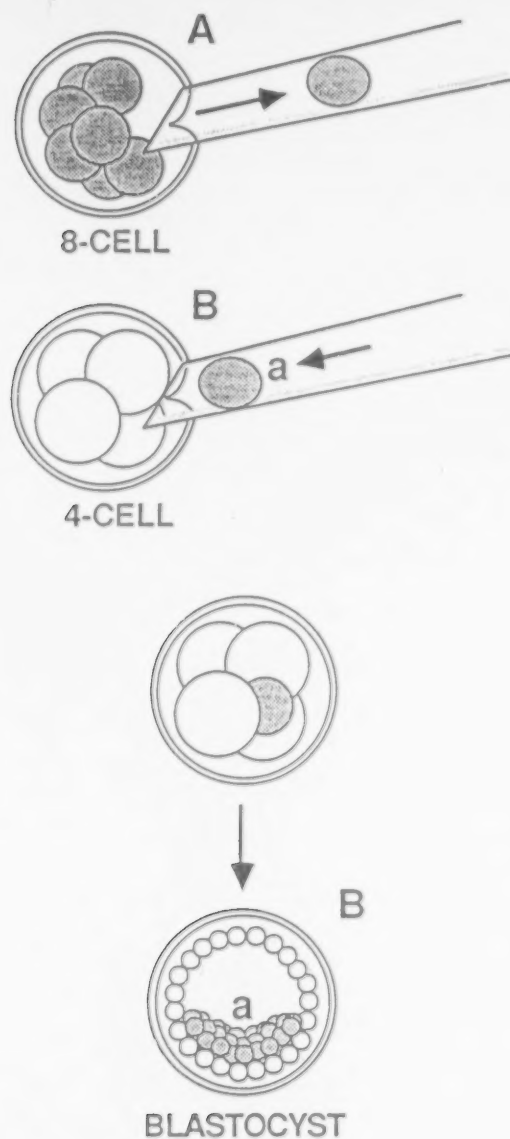


Figure 8

were collected surgically from the donor, micromanipulated and then were placed into the reproductive tract of intermediate host sheep until they advanced to the late morula or blastocyst stage. At this time, the developing embryos were removed from the intermediate host and surgically transferred to the respective host species recipients. This method of producing identical offspring in sheep, cattle and the horse (Willadsen and co-workers, 1980, 1981) is now considered a classical work. Using this approach, five surgeries are needed on at least four different animals to obtain one set of identical twin lambs from two recipient females. Unfortunately, this micromanipulation method was not practical enough to be used by the commercial livestock industry.

Splitting Embryos to Produce Twins.

Both monozygotic (one ovum) and dizygotic (two ova) twin offspring do occur in dairy and beef cattle. The incidence of twin births generally tends to be slightly higher for dairy cattle (1 to 7%) than for beef animals (0.5 to 5%). It has been estimated that only 2 to 10% of all twin calves born are the result of an embryo dividing into two similar parts during an early stage of gestation (monozygotic twins). It is likely that the occurrence of monozygotic twins in cattle is a random event rather than a hereditary trait. However, since dizygotic twins are the result of multiple ovulations in the female, genetic involvement of the dam is likely associated with the production of such twin births. The occurrence of a natural set of triplets (trizygotic) in domestic cattle is estimated to be 1:65,000 to 1:100,000 births.

In theory, it would seem that the production of twins from single-ovulating mammals (e.g. cattle) would be a potential method of improving

reproductive efficiency. This approach has some merit and has been evaluated extensively in several foreign countries (e.g. Ireland, Japan) that have a very small land mass for agriculture production. Seidel (1981) has indicated that approximately 70% of the nutrient intake of a beef cow is used for body maintenance and the remaining 30% of the nutrient intake is used for growth and maintenance of her calf during pregnancy. Consequently, cattle carrying twin fetuses would tend to be more efficient in feed utilization per calf produced and make better use of available grazing acreage.

In 1982, three research groups, one in France and two in the United States (Colorado and Louisiana) produced genetically identical twin calves by splitting single cattle embryos without animal surgery or an intermediate host to serve as an incubator (Ozil, 1982; Lambeth and co-workers, 1982; Williams and co-workers, 1982). Subsequently, more in depth studies with cattle morulae and blastocysts in Europe and the USA confirmed that bisecting embryos and transplanting resulting "half" embryos (demi-embryos) produced respectable transplant pregnancy rates in recipient females (Lambeth and co-workers, 1983; Ozil, 1983; Voelkel and co-workers, 1984c, 1984d; Williams and co-workers, 1984; Brem and co-workers, 1984; Baker and co-workers, 1984; Baker and Shea, 1985; Mertes and Bondioli, 1985). This new approach includes non-surgical transfer of micromanipulated embryos to recipient females with a standard AI catheter. The development of this new technique has made the production of genetically identical offspring by micromanipulation a reality to the cattle producers the world over. Embryo collection, micromanipulation and transfer to recipient females can be completed in a matter of an hour with this new technology.

The basic LSU embryo splitting procedure is performed with two microinstruments manufactured in the laboratory from soft glass capillary tubes. The first microinstrument consists of a suction pipette with a fire-polished tip. This pipette has an outside diameter slightly smaller than that of the embryo and is used to hold the embryo stationary during the manipulation process. The second microinstrument is a finely drawn glass surgical needle used to bisect the embryo. The day 6 to day 8 bovine embryo (diameter 120 to 200 microns) is generally not visible to the naked eye.

Individual embryos are held in position for microsurgery under Dulbecco's PBS holding medium by a 1 ml syringe (creating slight negative pressure) attached via plastic tubing to the suction pipette. The fine glass surgical needle is then used to mechanically remove the zona pellucida. Once the embryo has been removed from the zona pellucida, the surgical needle is used to bisect the embryonic cell mass (on a vertical plane) into halves (Lambeth and co-workers, 1983) (Figure 9a and 9b).

Each of the resulting "half" embryos can be either reinserted into the original zona pellucida or into another zona pellucida removed from an unfertilized ovum (e.g. cattle or swine). The "half" embryos, either one or both of them, are then individually drawn into .25 ml French semen straws for non-surgical transfer to recipient females.

Recently, Mertes and Bondioli (1985) suggested that using the glass surgical needle to bisect bovine embryos outside the zona pellucida (Lambeth and co-workers, 1983) might have an advantage over bisecting the embryo with a surgical blade using the basic procedure developed by Colorado (Williams and co-workers, 1982).

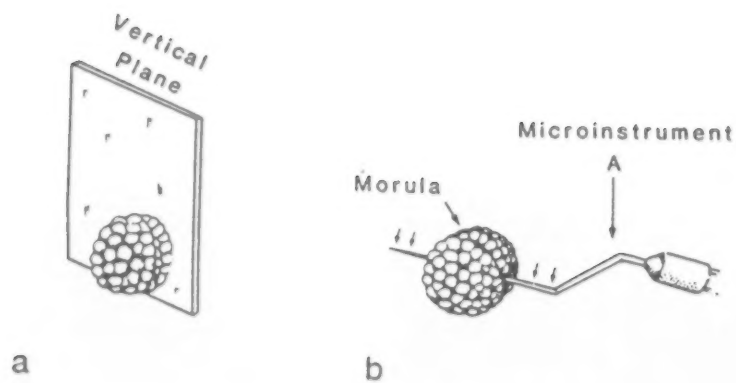


Figure 9. An early stage bovine embryo (a morula) is bisected on a vertical plane as shown in 9a and 9b. A fine glass microinstrument (surgical needle) is used to divide the embryo into halves (shown in 9b) while in holding medium (Lambeth and co-workers, 1983).

Since the birth of genetically identical twin calves from later-stage embryos in 1982, 650 or more split embryo transplant offspring have now been born to recipient cows in North America. Today, embryo splitting is being offered as a service to cattle producers by several commercial embryo transplant units in the United States and Canada. Usually, a set charge ranging from \$25 to \$150 is placed on each embryo that is microsurgically divided into two halves for subsequent transfer.

The results to date indicate that usually 45 to 65% of the "half" embryos transferred to good quality recipients result in pregnancies. Correspondingly, results from transferring whole embryos range from 50 to 70%. However, when these results are evaluated from the standpoint of number of transplant calves produced per number of embryos collected, 200 good quality whole embryos may result in 140 transplant calves born, whereas, 200 similar quality embryos divided into halves would yield 400 "half" embryos, which then may result in 220 split-embryo calves born or 110% pregnancy rate from 200 embryos.

Subsequent to the birth of identical twin calves from this new embryo splitting procedure, the first genetically identical twin offspring from sheep split embryos (morula and blastocyst stages) were reported from England (Willadsen and Godke; 1984) and Ireland (Gatica and co-workers, 1984) and more recently in Louisiana (McFarland and co-workers, 1985a). In addition, Tsunoda and co-workers (1984) have reported split embryo goat transplant twins born in Japan and more recently in the United States by Pendleton and co-workers (1986b). In June of 1984, the first set of identical twin foals resulted from a single split embryo (blastocyst stage) in Colorado (Slade and co-workers, 1984a; CSU Equine Sciences Newsletter, Vol. 3, 1984), and the first swine split-embryo

transplant litter was born in Louisiana in August of 1984 (Rorie and Co-workers, 1985a).

Producing embryo-engineered twin calves from early or later-stage embryos will result in genetically identical offspring of the same sex. For cattlemen this would remove the concern for freemartinism, if both "half" embryos were transferred to the same recipient female. In the initial studies with cattle, both micromanipulated "half" embryos were transplanted into the same recipient animal, however more recently, a single "half" embryo has been placed in each recipient female. In this case the twin set would be produced by two different surrogate mothers. It is interesting to note that pregnancy rates have been similar when either a single "half" embryo or two "half" embryos have been non-surgically transplanted into suitable recipient females.

We have noted at our station that the genetically identical split-embryo calves do not always have identical coat-color patterns. This has also been brought to our attention by concerned cattlemen and other researchers who have had sets of split embryo twins born from micromanipulated bovine embryos. Recently at the NAAB meeting in Denver, Seidel (1985) confirmed these observations and suggested that coat color patterns form individually as the transplant fetus develops in the uterus of the recipient female (an epigenetic effect). Since the melanoblasts migrate as a group, the coat color patterns of identical twins (e.g. Holstein) would be very similar, but likely not identical. Even though coat color patterns of these twins may not be phenotypically identical, split-embryo twin calves are considered to be genetically identical.

The importance of embryo splitting for mammals may have a far-reaching impact on the seedstock industry in the years to come. The

possibility exists that one-half of a micromanipulated embryo could be transferred to a recipient animal and the remaining half of the same embryo could be frozen for use at a later date. If the offspring derived from the first "half" embryo was of the quality the owner desired, the remaining "half" embryo could then be thawed and transferred to another recipient animal to produce the remaining twin offspring or possibly marketed as a sexed embryo of known quality. The possibility exists that this embryo splitting technology could be used in IVF clinics to help infertile couples. In a situation where only a few good quality embryos are produced these embryos could be bisected to produce demi-embryos. If the demi-embryos were frozen for transfer during subsequent cycles, this should markedly increase the number of chances one would have to produce an IVF baby.

Also, cattle have produced live offspring following embryo transfer of frozen-thawed "half" embryos (Lehn-Jensen and Willadsen, 1983). This technology may offer numerous opportunities to expand progeny testing of full sibs, methods for evaluating undesirable recessive traits, studying maternal uterine influence on offspring and performance testing of sires.

Recently, later-stage embryos (late morulae) from beef donor cattle (Louisiana) were split into "half" embryos then carefully dissected in quarters by microsurgery. In February of 1984, the first live "quarter" embryo calves were produced from separate surrogate mothers (Voelkel and co-workers, 1984e, 1985). All four "quarter" embryos were transferred non-surgically and two live genetically identical twin female calves were born.

In an effort to improve the efficiency of the embryo splitting procedure, Rorie and co-workers (1985b) in this laboratory have developed a simplified, less costly technique for bisecting bovine embryos. With this simplified procedure, a double-edged razor blade is used in place of the microinstruments and the commercial micromanipulation unit for making "half" embryos from later-stage farm animal embryos. The LSU razor blade technique can be performed on either morulae or blastocysts (e.g. day 6 to day 8 bovine embryos) with intact zonae pellucidae or with embryos with zonae removed with a pronase solution prior to bisection (McFarland and co-workers, 1985b). Results to date indicate embryos halved with the standard razor are as viable as those control embryos bisected with a fine glass needle. Using a razor blade for bisection could make the embryo splitting procedure practical and less costly for cattlemen already participating in an embryo transplant program.

In a recent study, Voelkel and co-workers (1984c) reported that when later-stage bovine morulae and blastocysts were bisected into "half" embryos, they did not have to be replaced into an empty zona pellucida before being transferred to recipient cattle. Results to date indicate that zona-free and zona-encased demi-embryos produce similar transplant pregnancy rates in beef cattle recipients.

It is interesting to note that occasionally when a later-stage bovine "half" embryo is non-surgically transferred either with a poor quality zona pellucida or without a zona, it divides producing a set of genetically identical twins in of itself. During the last two years this has happened four times at our laboratory. In these cases, three transplant offspring developed from a single pair of twin demi-embryos

(one "half" embryo transferred to each of two recipient females). If "half" embryos (later stage morulae and blastocysts) can be transplanted without a zona pellucida, as recently shown in this laboratory, the existing embryo splitting procedure would be made even more simplified and practical for on-the-farm use by cattle producers.

The Production of Chimeric Embryos.

Recently, efforts have been made with farm animal models to incorporate the embryonic cells (blastomeres) of two different embryos together in vitro to form one embryo that could then have up to four different parents. Offspring produced from this mixing together of blastomeres of two or more embryos would be termed a chimera (allophone). In 1982, Willadsen (England) verified his earlier studies (Willadsen, 1980, 1981) and that of Tucker and co-workers (1974) showing chimera offspring could be produced by aggregating blastomeres from two or more sheep embryos (e.g. from two or more breeds) into one composite chimera embryo. The offspring produced following the transfer of these embryos are then called intraspecies chimeras. More recently in the USA, later-stage chimeric sheep embryos (two breed types) have been constructed by either blastomere aggregation (Viker and co-workers, personal communications, 1984b) or inner cell mass microinjection techniques (Butler and co-worker, 1985) to produce viable ovine transplanted offspring. Intraspecies chimeras have also been produced in smaller laboratory mammals, including specific strains of mice, rats and rabbits (see review by Rossant and co-workers, 1982).

Preliminary observations on the early stages of life of the intraspecies chimeric farm animals in this laboratory suggest they may have

some growth potential over individuals of either of the two parent breeds. These observations need to be verified. This area of research appears to offer a multitude of opportunities to study and evaluate animal development, growth patterns and immunology in the years to come.

Recent research confirms that embryos of two different donor animals from two different species (sheep and goat) can be aggregated into one embryo to produce an animal that would be an interspecies chimera. The first viable sheep/goat chimera (male) was reported in England in 1984 (Fehilly and co-workers, 1984). West German researchers have recently duplicated this feat with sheep/goat embryos (Meinecke-Tillmann and Meinecke, 1984). In 1981, Willadsen (England) produced the first chimeric bovine offspring using blastomeres from embryos of two cattle breeds. Attempts have also been made to produce Bos tarus/Bos indicus chimeric calves using the inner mass cell injection technique (Summers and co-workers, 1983). Although seven transplant calves were born in this experiment, none were overtly chimeric, however, one calf did have internal tissues that gave some evidence of being chimeric in nature.

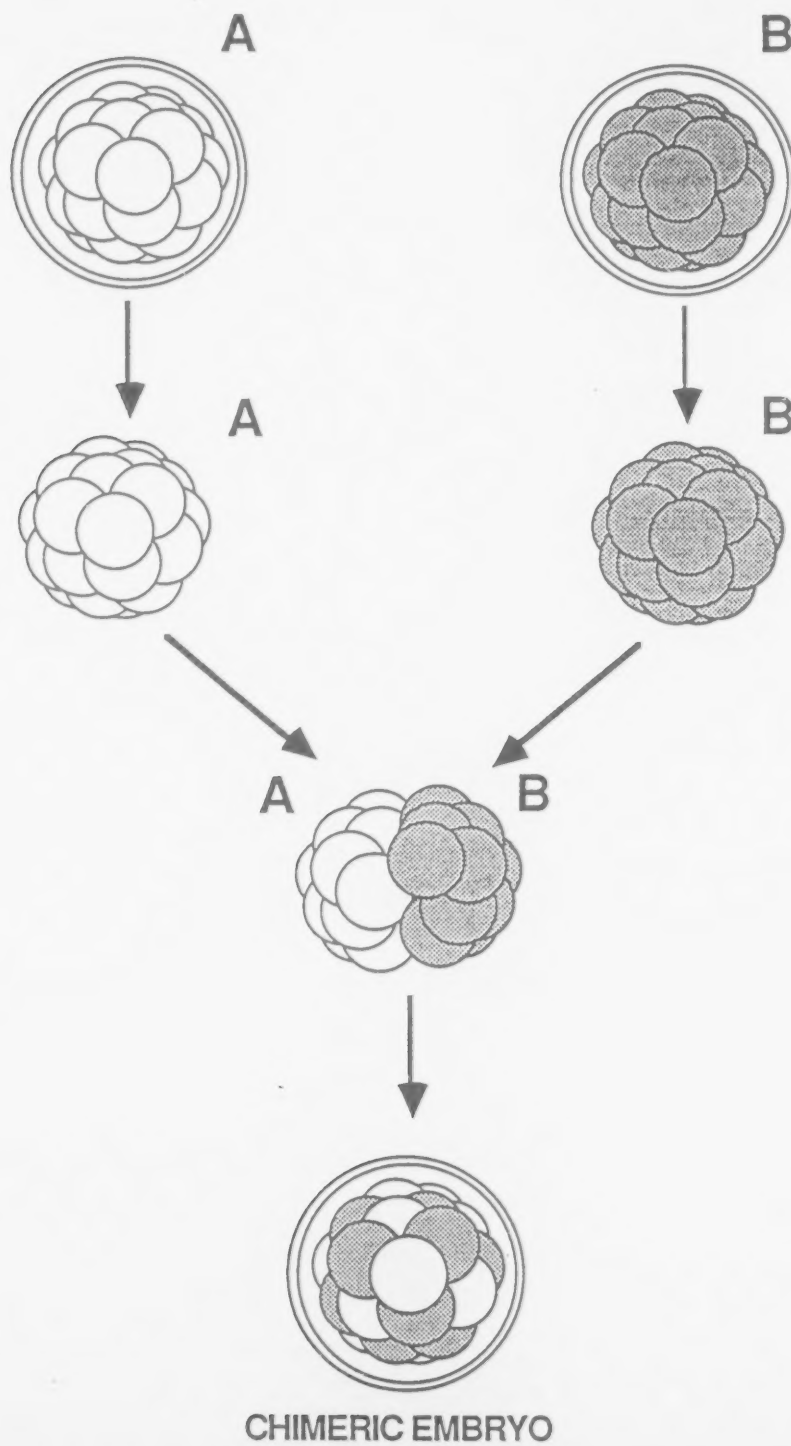
More recently, Brem and co-workers (1985) reported the birth of viable chimeric offspring by aggregation of later-stage bovine embryos of two domestic cattle breeds. One calf born had evidence of an overt chimeric phenotype. Recently, Rorie and co-workers (1986) in Louisiana produced a set of transplant twins from two different portions of two split beef cattle embryos (Figure 10). These twins had color patterns that were overtly chimeric in nature.

Inter Cell Mass Transfers Between Embryos.

A technique being developed for farm animals in Cambridge, England is the transfer of the cells that form the embryo (inner cell mass

Figure 10

AGGREGATION CHIMERA

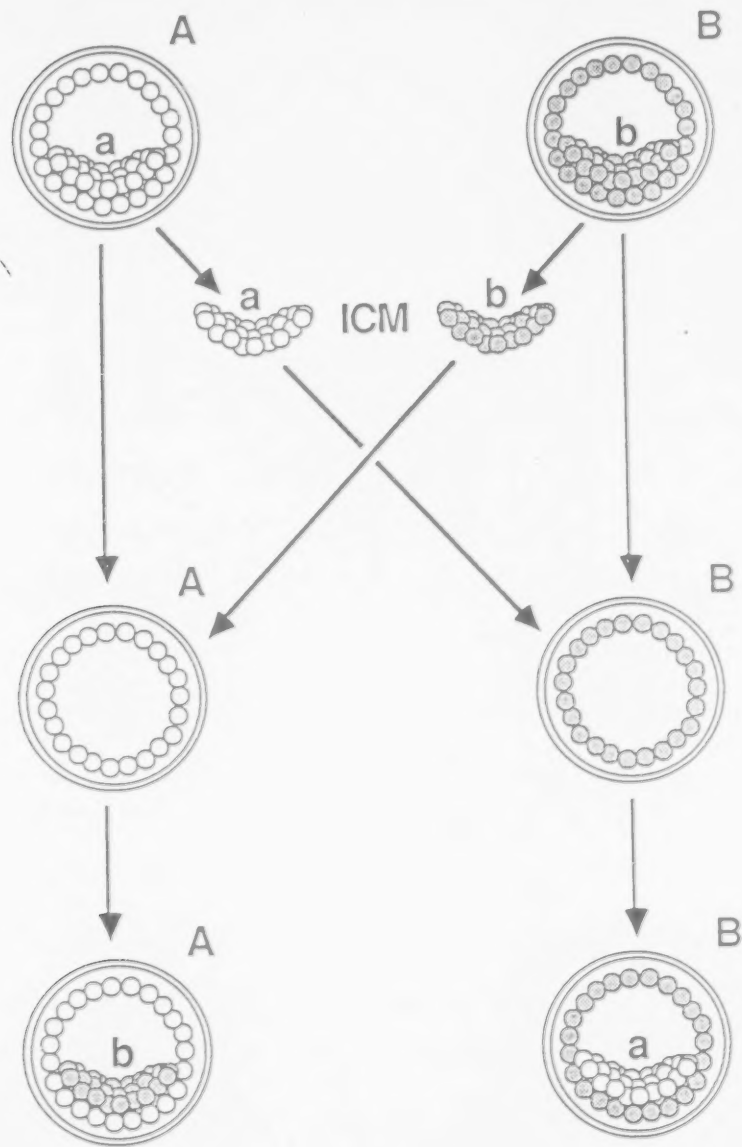


cells), using micromanipulation techniques, to another embryo of that species or to another species. If this is successfully completed, the embryo that has received the inner cell mass cells (by microsurgery or microinjection) will develop normally when transferred to a recipient female. The beauty of this micromanipulation technique is that the surgically altered embryo will have the placental membranes develop from the trophoctoderm of the second embryo but will have the fetus develop from the inner cell mass of the first embryo. This would allow the genetically engineered embryo to be placed in a recipient female of the second embryo's breed type (or species) to enhance an optimal environment for placental membrane attachment. However, the developing fetus would originate from the first embryo (see Figure 11).

A good example of this would be to use the sheep and goat (two different animal species) as embryo donor females. When a sheep embryo is transferred to a female goat recipient or a goat embryo is transferred to a female sheep recipient the transplanted embryos do not develop to term. If the inner cell mass of the sheep embryo was microsurgically removed and placed in the goat embryo the composite embryo could then be transferred to a goat recipient. The chance for an offspring to be born from this composite embryo is greatly enhanced since the placental membranes that develop from this embryo would be derived from the original goat embryo. In this procedure, the goat inner cell mass could then be placed in the remaining trophoctoderm cells of the sheep embryo and this composite embryo could be transferred to a female sheep recipient. The end result would be an interspecies transfer of two embryos with the goat giving birth to a lamb and the ewe giving birth to a goat offspring.

Figure 11

RECIPROCAL ICM TRANSFERS



The use of this new technique could be put to use in saving some of our endangered species, where more abundant recipient species could carry the embryos of endangered species to term. Examples might be — rare Indian Gaur cattle embryos placed in domestic cattle or embryos of the rare Przewalski's horse placed in domestic mare recipients. Requests are being put forth by zoological societies the world over to help them develop techniques using the inner cell mass transfer procedures to engineer embryos of exotic mammals.

Embryonic Cloning.

A clone is defined as a genetically identical offspring derived from transplanting the intact nucleus ($2n$) from a somatic cell of one individual into another individual (usually an embryo). For decades scientists have dreamed of producing identical animal clones. The ability to clone embryos through successive generations of identical clones would provide potent method for accelerating genetic change that could be more effective than artificial insemination.

In classical experiments clones of frogs have been produced by transplanting nuclei of somatic cells into enucleated, unfertilized frog eggs (Gurdon, 1964; King, 1966; Gurdon, 1977). The resulting offspring were similar phenotypically and the same sex as the nuclei donor.

The prospect of duplicating this feat in mammals was improved a slightly when Bromhall (1975) reported some success in vitro with the experimental introduction of nuclei from the embryo of a rabbit into an unfertilized rabbit ova by a microinjection procedure and virus-induced fusion. Although success has been reported with several more primitive animals (e.g. amphibians), it was thought to be not possible in warm-blooded mammals.

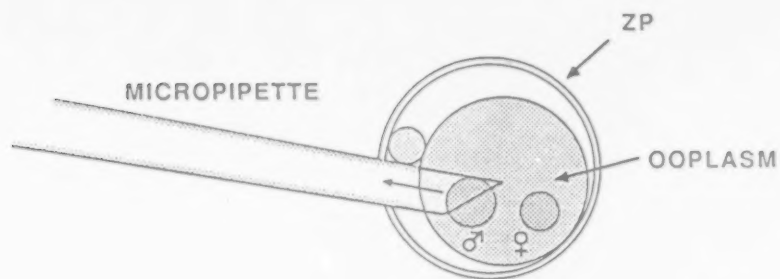
In 1981, a Swiss and an American scientist reported producing the first cloned mammals (mice) using embryonic nuclei microinjection techniques (Illmensee and Hoppe, 1981). This first report of live births has caused a great deal of controversy among some scientists, but stimulated a wealth of creative thinking among others.

The approach used in these initial cloning studies was to extract nuclei ($2n$) from the cells of the inner cell mass of a developing mouse embryo and transplant them into a sperm-activated mouse ova, using microinjection techniques. Using a microsuction procedure the male and female pronuclei of the activated host ovum are removed leaving the transplanted nucleus ($2n$) in the activated ovum to develop into a cloned embryo (Figure 12). Offspring produced by this method would be clones of an embryo, not clones of an adult. Cloning adult somatic cells would be the ultimate end point of making this procedure applicable to the livestock industry.

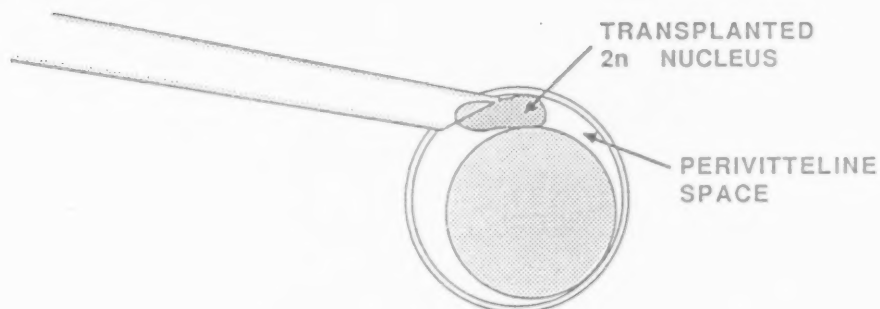
With the controversy over the mouse "clones" of Illmensee and Hoppe (McGrath and Solters, 1984; Markert, 1984 and others), many researchers concluded that embryonic clones in farm animals would remain as just a dream to scientists. Then, Willadsen (1986) reported the birth of the first nuclear transplant sheep offspring born in the world from his Cambridge laboratory. With this procedure, whole blastomeres from 8-cell and 16-cell ovine embryos were combined with either enucleated or nucleated halves of unfertilized ovine ova. Single blastomeres were fused with prepared ova halves using either electrofusion or Sendai virus. The reconstituted "half" ovum was then embedded in agar and cultured in vivo in a ligated oviduct of a diestrus ewe (Willadsen, 1979).

Figure 12

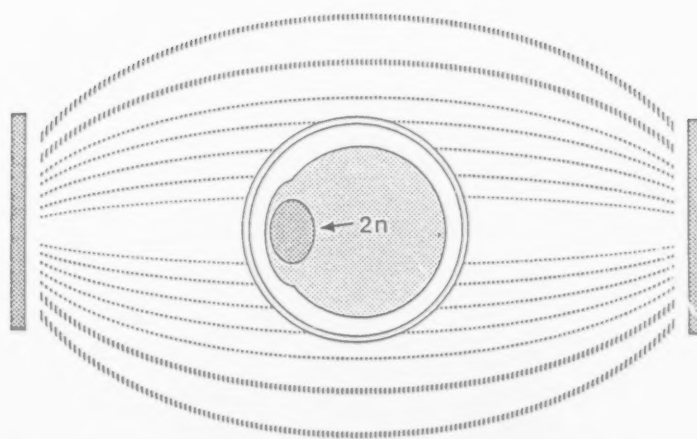
EMBRYONIC CLONES



REMOVAL OF MALE AND FEMALE
PRONUCLEI FROM PRONUCLEAR EMBRYO



INJECTION OF 2n NUCLEUS



ELECTROFUSION

The greatest number of reconstituted embryos blastulating following culture (48.3%) occurred when 1/16 of a 16-cell embryo was combined with electrofusion to an enucleated 'half' ovum. When reconstituted blastocysts were transferred to sheep recipients, three of the four developed to full-term lambs. All three lambs (two of which were identical twins) were the result of a blastomere from an 8-cell embryo combined with an enucleated "half" ovum.

Recently, newsline services in November have reported that Steen Willadsen has produced the first live nuclear transplant calves while a visiting scientist at Granada Genetics in Marquez, Texas. The news release indicated that several calves had been born from the genetic material of one bovine embryo. The genetic material from 8-cell embryos was placed in unfertilized eggs (without destroying them) and then later the embryos were transferred into surrogate mothers. Granada Corporation's spokesman Dr. Darold McCalla indicated in the news release that the details of this research was not yet reported in the scientific literature. Other research laboratories in the United States are working on developing nuclear transplantation techniques for swine and cattle. Other nuclear transplant offspring will likely be born and reported within the next 2 years.

The potential for the use of somatic cloning to improve production of farm animals in the years to come is almost impossible to imagine. Let's use as an example the top milk producing dairy cow in North America. Somatic cells from this exceptional cow could become the pool of genetic material to clone hundreds of new offspring that all have the same genotype of that top milk producing cow. The nuclei of these somatic cells could be extracted and placed in fertilized ova of lower

producing cows. The genetic material in the host ova would be removed and discarded allowing the embryonic development of a genetically engineered embryo to be under the control of the transplant nuclei.

The impact on milk production in one herd alone that had clones of the top producing cow as replacement females would be phenomenal to say the least. The genetic material of top producing animals throughout the world could be made available to virtually any livestock producer with this adult cloning technique. It appears that the future for genetic engineering in this area has incredible economic potential. With these types of new developments in genetic engineering, supporting research with an application for increasing world food production would be an investment in the future of mankind.

DNA (Gene) Injection to Produce Transgenic Animals.

This area of research surfaced in 1981 and has subsequently become one of the leading developmental areas for commercial genetic engineering firms in the USA. The basic approach to gene injection involves taking genetic material (genes) and, with microinjection techniques, placing this material (usually synthesized or from another species) near or into the pronuclei of recently sperm-penetrated ova in the process of fertilization (see Gordon and co-workers, 1980). These DNA-injected embryos are then transplanted into recipient animals. If the DNA material becomes incorporated into the $2n$ nucleus of the 1-cell zygote, the resulting transplant offspring then would have the potential of genetically altered capability at some time during their own life.

From a classic study in 1981, it was reported that a DNA sequence (gene) controlling the production of hemoglobin in the rabbit was

injected into the male pronuclei of recently sperm-activated mouse ova. When the transplant mice were born they were able to produce rabbit hemoglobin in addition to mouse hemoglobin for their red blood cells. Even more important to the future of this transgenic technique was that some of the natural offspring of these genetically engineered mice were able to produce rabbit hemoglobin, implying that this was a heritable characteristic for these mice (Wagner and co-workers, 1981). More recently, Wagner and co-workers (1984) have suggested that genes could be introduced into fertilized ova of farm animals to produce transgenic embryo transplant offspring.

Many new genetic engineering companies are working to isolate and identify specific genes and then duplicate them in the molecular biology laboratory. Scientists are now looking into ways of inserting these man-made genes into activated ova and embryos of farm animals. One gene of interest to researchers at present is the Growth Hormone (GH) gene, and the possibility of incorporating it into swine, sheep and cattle ova. If the genetic material introduced into the these activated ova becomes incorporated in the nuclear DNA, the possibility exists that the GH gene could be activated to stimulate rapid growth, as these transplant offspring develop toward maturity. Successful incorporation and expression of the GH gene has already been reported in embryo transplant mice (Palmiter and co-workers, 1982; Palmiter and co-workers, 1983 and others).

It is interesting to note that daily injections of GH has been reported to result in up to 15% increase in milk production in dairy cows (see Bines and co-workers, 1980; Peel and co-workers, 1981). If the GH gene could be incorporated into the nuclear material of dairy cattle

embryos, then the possibility of increased milk production would exist, if gene expression occurred during lactation. This could then eliminate the need for daily exogenous administration of GH for the lactating dairy cow. Research is needed to explore this possibility.

The potential for other genes to be used in farm animal genetic engineering research are now being identified and evaluated. Davis and co-worker (1982) have described the ovine Booroola gene that affects ovulation rate in sheep. When this gene is present and expressed in the Australian Merino ewes, ovulation and multiple lambing rates increase in the flock. In a more recent report, another gene(s) has been identified that increases ovulation rates up to six fold in mice (Spearow, 1984). Possibly, the Booroola gene could be incorporated into ovine embryos of single-ovulating sheep breeds in hopes of increasing ovulation rates of ewes in subsequent generations. The Spearow or similar genes should not be overlooked for their potential to increase the ovulation rate and the number of offspring produced in single and litter bearing animals.

The first large scale effort to produce transgenic livestock was started by Dr. Kevin Ward and his group at CSIRO in Sidney, Australia. Their approach was to incorporate a GH gene in sperm-activated ovine ova to potentially increase the growth rate in sheep transplant offspring. Although gene-injected offspring were born in their initial study, unofficial reports indicate that none of the transplant lambs to date have been found to have GH genes incorporated in their somatic cells or have given evidence of gene expression.

The first transgenic farm animal offspring were reported by Hammer and co-workers (1985). Pronuclei of activated ova from rabbits, pigs and sheep were microinjected with copies of the human GH gene which had

been fused to a mouse regulatory gene (MT-hGH). Of the transplant offspring born, 1.3% of the 73 lambs, 10.4% of the 192 piglets and 12.8% of the newborn rabbits were found to have some level of gene integration. When MT-hGH mRNA was monitored, 4 of 16 rabbits and 11 of 20 pigs had a positive response to the gene expression test. Gene expression was not detected in the gene-injected transplant sheep.

Dr. Duane Kraemer and his group at the Texas A&M University have produced what are believed the first gene-injected bovine transplant offspring (cited in the Gulf Coast Cattleman, January, 1986). In this study, genes were microinjected in the male pronucleus of sperm-activated bovine ova. Six transplant calves have been born to date, with only one of these calves having the possibility of being transgenic. Further evaluation for potential gene incorporation is presently underway. Several research groups have gene-injected embryo transplant pregnancies now underway in swine, goats and cattle in North America and are waiting for offspring to be born. Recently, Fabricant and co-workers (1987) have produced gene injected transplant goat offspring, but DNA incorporation could not be established.

This area of research is but one example of many marked advancements now being made in the field of animal reproductive physiology. There appears to be a very short lag-time between discovery and implementation in this area, especially in animal agriculture. In the months and years to come, additional animals will likely be produced that will express incorporated genes, and offspring of other species may be produced that are true embryonic clones. Animal reproduction is truly entering a new era; the genetic engineering revolution is apparently underway for both man and animals.

PART II
Progress
and
Potential Application of Animal Reproductive
Technologies
to Humans

PART II.

Introduction

Human Infertility Problems.

It has been estimated that one in every six Americans face some type of human infertility problem, and this number is on the increase in our modern day society. Well over 10 million men in North America are now estimated to have low spermatozoa concentrations and some two million women in the USA have difficulty getting pregnant. Of these women, now more than 500,000 infertile females both young and old are estimated to have uterine tubal problems, most of which are untreatable even with modern medical technology. This leads to the estimate that 10% of the American couples are trying to cope with some type of infertility problems, and from all the indications the percent is on the increase in recent years. The reason for the trend for increased human infertility problems is not clear. However, sociologists are now suggesting that the "working woman" and the stress of modern society are leading to individual anxiety and sexual dysfunction in the male and/or female.

With many of the women now working in our society the pregnancy pattern for these females has been gradually changing over the last 10 years. Pregnancy rates for women 30 years of age and older have almost doubled in the USA. The most fertile years for females in North America are from ages 20 to 30, then there is a decline to the age of 40. Normally, 70% of the women attempting to become pregnant, do so within the first year. Correspondingly, 5% of the women attempting to become pregnant do so after the first year prior to the end of the second year.

Often two years or more are now needed for females to conceive when women approach their mid-thirties. These women that fear early menopause, face the stress of working and have anxiety of wanting children have emerged a dominant group in our society having infertility problems.

The stress of economic survival in the working community is apparently taking its toll on the reproductive capacity of the working man also. A notable increase in men complaining to their doctors of sexual dysfunction has also emerged in our society in recent years. This overall situation has caused society to search for technical assistance from the medical profession. The medical profession only recently has been evaluating new technologies and approaches in attempt to deal with the new demands from society.

Part II.

TABLE 7. PROGRESS IN REPRODUCTIVE TECHNOLOGIES PRIOR TO 1987¹

Technology x Mammalian Group	Laboratory Animals	Farm Animals	Humans	OTA Ranking ²
1. Non-surgical embryo transfer methodology	2	9*	1	3
2. Gonadal tissue transplantation	2*	2*	1	2
3. Superovulation methodology	9*	8	4	3
4. Maturation of oocytes <u>in vitro</u> (live young)	1	2	8*	3
5. Methods for sexing sperm cells	1	2	4*	2
6. Treatment of spermatozoa to increase fertility	1	5*	2	1
7. <u>In vitro</u> fertilization methodology	2	2	9*	3
8. Sperm cell microinjection for fertilization	1	1	1	2
9. Methods for freezing embryos	6	9*	2	3
10. Methods for freezing oocytes	1	1	1	3
11. Methods for freezing and storage of sperm cells	2	8*	5	3
12. <u>In vitro</u> culture system methodology	5	7*	3	3
13. Methods for sexing embryos	4	6*	1	2
14. Splitting embryo procedures	4	10*	1	1
15. Production of chimeric embryos	3	7*	1	1
16. Procedures developed for inner cell mass transfers	2	6*	1	1
17. Methods for embryonic cloning developed	2	3*	1	1
18. Methodology for gene insertion	6*	4	1	2
a. Composite Score	= 54.0	92.0	47.0	
b. Mean Technology x Group	= 3.0	5.1	2.6	
c. Number of Top Rankings	= 3	11	3	
d. Number of Bottom Rankings	= 7	2	12	

¹Progress scores = 10 highest to 1 lowest based on information available.

²OTA priority rankings = 3 highest, 2 = moderate and 1 = lowest.

Part II. - Topic Areas

Developing Non-Surgical Embryo Transfer Methodology.

Progress.

The progress in developing surgical embryo transfer procedures for cattle, sheep, goats, swine and horses has been well documented. Procedures for the majority of the laboratory animals and several exotic species are also available (e.g. mink, ferret). Efforts have been made over the years to develop non-surgical procedures for embryo collection and transfer of farm animal embryos. Although there are several reports in the scientific literature of some success using this approach (e.g. sheep, goat), only in the cow and the horse have the non-surgical collection and transfer procedures been developed to the point of now being used in the commercial setting. The cow, for example, non-surgical embryo transfer procedures are now used routinely by commercial companies to produce an estimated 100,000 embryo transplant offspring annually in North America. One would expect a 60 to 80% non-surgical embryo recovery rate in the cow and a 55 to 90% non-surgical embryo recovery rate for the horse with these procedures.

Procedures have been developed to collect later-stage embryos non-surgically from the human (Croxatta et al., 1972) and the baboon (Kraemer et al., 1979, 1980; Pope et al., 1980). Using non-surgical methodology, Pope and co-workers have produce a pregnancy in the baboon and Bavister and co-workers (1985) have reported a transplant pregnancy in the Rhesus monkey. In 1984, Dr. Buster and his colleagues in California reported what is believed to be the first documented human embryo transfer (one female to another) using non-surgical collection

and transfer methodology. Others have followed, and its projected that many more human transplant babies will be born by the end of 1988.

Projections.

As society accepts the idea of human embryo transfer more of the human in vitro fertilization clinics now coming into existence in the USA will begin offering embryo transfer as a service for infertile couples. Since non-surgical embryo collection procedures requires that the embryo be in the uterus properly at the time of collection, human embryos will be collected at a later morphological stage of development and then transferred to surrogate females. Since pregnancy rates have been the highest transferring embryos at the morula to the blastocyst stage of development in most other mammals evaluated, it is likely these later-stage embryos will also become the development stage of choice for human embryo transfer in the future.

Since both non-surgical embryo collection and non-surgical transfer only takes a few minutes to perform, it is not unreasonable to propose that embryo transplantation in humans will likely develop as an out-patient service for both the donor and surrogate female. In the future, females with incurable diseases (e.g. cancer) will seriously consider human embryo transplantation to produce children.

Potential for Transplantation of Gonadal Tissue.

Progress.

Successful ovarian tissue grafts have been reported in laboratory animals (e.g. rats, hamsters) and farm animals (e.g. sheep and cattle). The best results from these experiments have occurred with autografts and grafts among animals of highly inbred strains or lines. The results

from attempts to graft testicular tissue grafts are less convincing. Testicular grafts have been reported in several species (e.g. roosters, frog, mice and rats). The primary problem in gonad transplantation relates to the histocompatibility between the tissue donor and the recipient. The best results for heterotransfer reported to date in larger animals was reported by researchers at Texas A&M (Griffin and Randel, 1978; Acosta and co-workers 1982), who successfully transplanted ovaries from beef cattle to unrelated recipient cattle without tissue rejection.

In humans, testicular autotransfers have been documented in the scientific literature (Silber and Kelly, 1976 and others). Several years later, Silber (1978) reported successful surgical heterotransfer of the human testis for treatment of anorchia. In this case, the donor (age 30) was the genetically identical twin to the recipient of the transplant. Some 8 months after the surgery the sperm count ranged from 8 to 15 million/ml, with a 30 to 50% progressive motility rating. Two years post-transplantation, the recipient twin and his spouse produced a baby boy (Silber and Rodriguez-Rigau, 1980).

Projections.

Preliminary results from several animals and the human suggest that ovary and testicle transplantation in humans may not be an impossibility, as once thought. The technical aspects of the microsurgery for this procedure appears not to be the problem. In fact, specific methodology for these procedures are already described in the scientific literature. The concern with gonad transplantation procedures is tissue rejection, which is much the same as with other organ transplants between individuals. With recent advances made with anti-rejection drugs (e.g.

cyclosporine), the possibility of gonad transplantation may actually become a reality in the future.

Research in this area is important to man in studying ovarian/testicular involvement in the human aging process, and possibly transplants could be used as a last resort effort for human infertility. The source of gonadal tissue, in the latter case, will likely be from the recipient's immediate family. One example, in this case, would a young woman who had to have a bilateral ovariectomy due to ovarian cystic disease or ovarian cancer. Her older sister (who already has her family) offers one of her ovaries to the woman to allow the victim to later have children.

In recent years, many young men have selected vasectomy as a means of contraception. Usually due to a grave family mishapening, a significant number of these men are now seeking medical assistance in hopes that someone will be able to reconstruct a viable sperm cell passage in their vas deferens to allow them to regain their fathering ability. In addition, continued efforts in developing genital reconstructive surgery and testicular transplantation methodology are important to be able to aid war-wounded veterans. There is a need for additional funded animal research in this area, since there will likely be good potential for technology transfer to humans sometime in the future.

Superovulation Treatments for Supplemental Oocyte Production.

Progress.

Procedures have been developed for superovulating laboratory animals, sheep, goats, swine, cattle and several exotic species. The gonadotropins used for follicular stimulation in these species have been

various dose levels of pregnant mare's serum gonadotropin (PMSG) and follicle stimulating hormone (FSH) from slaughterhouse pituitary tissues. Little success producing viable embryos has been reported with superovulation in the horse. Experimental results have been reported for animals using equine pituitary extracts (EPE) and human menopausal gonadotropin (HMG) and more recently trials have begun using a purified FSH and a recombinant-DNA FSH product.

Another approach to superovulation of farm animals has been to administer an anti-PMSG antibody along with PMSG to obtain a more controlled response during the superovulation procedure. The antibody supplementation approach has given encouraging results thus far in superovulating cattle. A newer approach to the superovulation of sheep and cattle has been to passively immunize the donors to various steroid hormones prior to the time of embryo collection. Preliminary results with sheep indicate that this approach has merit for a moderate increase in follicle development, and this method is presently being evaluated in cattle (Boland and co-workers, 1985). When androstendione is used to passively immunize a potential donor female (e.g. sheep) follicular atresia is reduced and reliable multiple ovulation results.

Efforts to stimulate follicular growth in the human female over the years have included various combinations of clomiphene citrate, HMG (pergonol) and human chorionic gonadotropin (HCG). These procedures have been well documented and several basic treatment schemes have now become standardized for most human IVF clinics. Results, for the most part, have been somewhat variable from female to female.

Projections.

With the advent recombinant-DNA FSH and LH products, it is likely that these gonadotropins will be evaluated in future for superovulation responses in the human female. A purified FSH or LH product of this nature will likely be less antigenic in nature over that of animal pituitary extracts (FSH-P) presently available on the commercial market for animals. As procedures are developed for the passive immunity approach to superovulation in animal models, it is not unreasonable to assume that this technology could be transferred to humans. Another approach to superovulation to be considered for the future is to administer an anti-inhibin antibody to women to allow natural multiple ovulations for ova collection procedures. Certainly additional research is needed in these areas to develop these methodologies using animal models.

Maturation of Oocytes with In Vitro Systems.

Progress.

A review of the scientific literature for laboratory animals reveals that fertilization of mammalian matured in culture is usually very low. Furthermore, a very few live offspring have been reportedly born from oocytes matured in culture, fertilized and transferred to recipient female. Cross and Brinster (1970) have produced live offspring from this approach in the mouse and Thibault (1972) has reported offspring in the rabbit. In 1985, Desjardins and co-workers reported successful maturation of equine oocytes in vitro using a modified Krebs-Ringer bicarbonate medium. Some recent success with maturation in culture has been reported with porcine and ovine oocytes and live

transplant offspring have resulted from this procedure (Cheng and co-workers, 1986). Recent advances have been made in evaluating the competence of harvested bovine oocytes following short-term oocyte culture (First and co-workers, 1986).

Oocytes collected from ovaries of non-living mammalian donors have been harvested many times over the years, but little culture success has been reported to date.

The oocyte maturation model developed for the mouse appears to be different than that of humans, since greater than 50% of the oocytes collected from a normal human ovary via laparoscopy are capable of being fertilized in vitro when exposed to good quality human spermatozoa in vitro. At this stage of development, it appears that human oocyte culture technology is more advanced than that of laboratory or farm animal species.

Projections.

In the future, research on oocyte culture systems will continue to develop a better understanding of various animal models. The technology developed from these studies will have the potential for transfer to the human oocyte culture system, which could ultimately enhance human in vitro pregnancy rates. Some success has been reported with oviductal organ culture systems in laboratory animals that should not be overlooked for human oocyte culture in the future. Furthermore, the possibility exists that the uterine or oviductal monolayer culture systems will become the basis of modern oocyte culture methodology. Recent advancements in hollow-fiber cell culture technology could offer an efficient method for oocyte maturation in the future. It is proposed

that semi-permeable membranes could be used to replace the zona pellucida during oocyte or embryo culture.

During the next 5 years efforts will be intensified to develop an optimal recipe of ingredients needed to gain in vitro oocyte competence in various mammalian oocyte models. Some evidence suggests that gonadotropins and steroid hormones may be needed for this process in some mammalian species. Possibly, unidentified oocyte maturation factors will be discovered to enhance this process. Without oocyte competence, in vitro fertilization for animals and humans can not be fully achieved.

In the future, progress will likely be made towards oocyte fusion and parthenogenesis of the human ovum. Also, much research will be generated on methodology for incorporating genetic material (genes) into the pronuclear portion of the sperm-activated human ovum or possibly the human embryo. Progress can not be made in either of these area without an effective oocyte culture system to sustain ova viability.

The Potential for Sexing Sperm Cells.

Progress.

Many scientific articles appear in the scientific literature on attempts to sex X and Y spermatozoa. Most of the experiments have been conducted on semen of laboratory animals, with the domestic rabbit being the animal most often used in these studies. Such approaches as centrifugation, separation by electrophoretic methods, migration in cervical fluids and atmospheric pressures are among the many different avenues evaluated over the years. Although individual experiments using some of these approaches have given encouraging results, usually the findings could not be repeated at a later date in the same laboratory or other

research units. In most cases, the number of observations ("sexed" offspring born) used in these experiments were too small to establish a true sex ratio change in an animal population. After evaluating the scientific literature, most scientists agree that the overall semen sexing results reported were not very consistent and most often were not convincing. It would be of great economic benefit to commercial livestock producers to be able to sex semen of farm animals. Various researchers groups have attempted this feat in recent years, but at present there is no scientifically proven procedure or kit on the commercial market.

In humans, the most recent approach to sexing semen involves the use of bovine serum albumin (BSA) to separate the X- and Y-bearing spermatozoa (Ericsson and co-workers, 1973). According to the theory behind this procedure, the sperm cells that have successfully migrated the BSA gradient should result in a Y-rich sperm cell fraction, which is then used for artificial insemination. Generally, positive results using this approach to sexing human semen have appeared in the scientific literature. In 1984, Ericsson reported in the popular press (Time Magazine, August 1984) that of 146 women who became pregnant using semen sexed by this method at clinics licensed by his Gametrics Company, 112 females gave birth to male offspring, for a 77% success rate. Gametrics, Inc. now has licensed clinics established across the USA and in countries outside of North America. However, some members of the scientific community still remain a bit skeptical of this semen sexing technology.

Projections.

The interest in separation of the X- and Y-bearing spermatozoa will continue to draw researchers to this research area for years to come.

Whether any of the older approaches to semen separation (e.g. centrifugation) are ever verified, so that the technology can be transferred to human medicine remains to be seen. At this stage of development, it appears that the BSA separation procedure for human sperm cells is more advanced than it is for sexing semen in animals.

A different approach to sex preselected offspring involves exposing sperm cells to H-Y antiserum (monoclonal antibody) at the time of artificial insemination. Zavos (1983), using the rabbit model, has produced encouraging results using this new procedure. With this approach, the H-Y antibody attaches to the male-producing sperm cells, which then allows the X-bearing sperm cells of the semen a greater opportunity for fertilization of the ova in vivo. If the results of this research prove to be worthy in animal models it would be reasonable to assume that this technology could be easily transferred to human reproductive medicine.

At present, several laboratories are trying to develop monoclonal antibodies that will detect specific antigens on male-producing sperm cells to use in an in vitro system to separate X- and Y-bearing spermatozoa. Also, one should not overlook the possibility of H-Y antigen immunization of women as a possible method of sex preselection in humans in the future. Researchers are now proposing that progress in the immunology area will likely be fruitful in human reproductive medicine during the next 5 years.

In Vitro Fertilization Procedures.

Progress.

In Vitro fertilization has been attempted in mammals for nearly a century. Prior to 1975, in vitro fertilization of mammalian ova had

been reported in more than ten different species. However, when the criteria for successful in vitro fertilization (IVF) for laboratory animals includes the birth of live offspring of both sexes after transfer to recipient females, the rabbit (Chang, 1959), the mouse (Whittingham, 1968) and the rat (Toyoda and Chang, 1974) are the only species prior to 1980 that have had successful in vitro fertilization. In other laboratory animal species IVF pregnancies have been established, but live births did not result or were not reported.

Attempts to produce viable offspring from farm animals with a complete in vitro fertilization procedure prior to 1980 were not successful. The world's first in vitro fertilized calf was produced in the United States in 1981 (Brackett and co-workers, 1981) and this feat has been duplicated by several laboratories since 1983. The only known IVF offspring produced in the world from both swine and sheep ova were produced at the ARC Institute of Animal Physiology in Cambridge, England (Cheng and co-workers, 1986). Recently, Japanese scientists have reported the first IVF goat offspring born, however, no IVF offspring have been reported to date in the horse.

In humans, the first birth recorded in the world in vitro fertilization occurred in July 1978 in England (Steptoe and Edwards, 1978). The second IVF birth was reported in India and many more have followed. Today, human IVF clinics are operating in many countries of the world, including some 150 or more in the United States. It has been estimated that over 1,000 IVF babies have now been worldwide.

Although the IVF technology has become commonplace in human medicine, it is interesting to note that only limited success with embryo development has been reported in the scientific literature for non-human

primates. The most notable success story was when the first IVF baboon offspring was reported by Clayton and Kuehl (1984). The reason for the poor success rates with IVF procedures in non-human primates to date can not be explained.

Projections.

In this area of reproductive physiology, the evidence clearly indicates that the technology available in human reproductive medicine is more advanced than any of the animal species in which in vitro fertilization has been attempted. For infertile couples, IVF technology now offers a new ray of hope over that of going through the standard child adoption procedures. IVF procedures have been most beneficial to those couples where the male is considered to be fertile and the female has oviductal problems, but is otherwise normal. With human IVF the genetic parents are also the gestational parents, which is generally more acceptable to couples than the child adoption approach.

In the future, it is predicted that virtually every major hospital will have an IVF clinic to assist infertile couples. The in vitro fertilization procedure will become an integral part of human embryo transplant programs that are projected to develop in the Europe, Australian and North America during the next 5 to 10 years. In addition to the frozen sperm banks already in existence for humans, frozen ova banks will likely develop for use by infertile couples.

The possibility exists that sperm microinjection techniques will be used to more efficiently fertilize human ova in the future. Studies presently underway with farm animal embryos may add the new technology that would increase the percent fertilization of human ova. The addition of heparin (Parrish and co-worker, 1985, 1986), glycosaminoglycans

(Lenz and co-workers, 1983), chondroitin sulfate (Lenz and co-workers, 1983) or possibly bovine serum albumin (Glaub and co-workers, 1978) to the semen as a pre-treatment to increase the fertilizing potential are some possibilities that could be evaluated in the human IVF system. Also, improved embryo culture procedures, such as the use of the monolayer culture system or the addition of trophoblastic vesicles to the culture medium are real possibilities for the future. The use of sexed semen for human in vitro fertilization may be a reality sometime in the future.

Fertilization of the Ovum by Sperm Cell Microinjection.

Progress.

Many attempts have been made using sperm microinjection procedures in laboratory animals, but with little success at producing offspring. Using this approach fertilization and cleavage to 2-cell embryos usually is less than 20%. For farm animals, only two calves have been reported in the scientific literature from using sperm cell microinjection (Meryman, 1960; Graham and co-workers, 1974). In a recent review, Iritani and co-worker (1984) projects that sperm cell microinjection techniques will develop more rapidly in the coming years for farm animals, due to better understanding of the biochemistry of the mammalian ovum and improved microinjection procedures.

Thus far in humans, no offspring have been reported in the scientific literature that have been the result of microinjecting human sperm cells into an ovum.

Projections.

Although little success producing offspring has resulted from sperm cell microinjection procedures to date, this remains as an area of great

interest to researchers. New microinjection techniques and electro-fusion technology offers an improved chance for success in the area of research. For humans males with low sperm concentrations, sperm microinjection technology could offer a viable alternative low fertility. Since only a single sperm cell would be needed to fertilize the human ovum with microinjection, a male with only a few viable sperm cells per ejaculate could father a child.

The possibility still exists that freeze dried sperm cells could be used in this artificial fertilization procedure. A recent report suggests that the DNA of freeze dried spermatozoa remains intact and potentially viable. The need for motile sperm cells, under natural fertilization conditions, is to penetrate the zona pellucida. Sperm cell microinjection through the zona pellucida should remove the motility requirement usually needed for the fertilization process.

Recent efforts in fertilization research is being directed towards oocyte fusion to produce a zygote. Some years ago, Soupart and co-workers (1978) reported some success fusing one oocyte with another to produce an embryo. Unfortunately, none of the embryos in this study continued developing to term. This research area is presently under investigation using oocytes of laboratory animal species as well as farm animals. This approach would certainly offer a unique alternate to an infertile couple where the male is the primary problem. Before a great deal of progress can be made on oocyte fusion, additional basic research is needed on the mammalian ovum.

Freezing and Storage of Mammalian Embryos.

Progress.

The first live offspring born from frozen-thawed mammalian embryos (mice) was first reported in England (Whittingham, 1972). Since then similar procedures have been developed to successfully freeze embryos of other laboratory animal embryos. The technology for freezing mouse embryos is now used as the embryo freezing model for most other mammals. Studies thus far suggest that embryos frozen and stored in liquid nitrogen at -196°C remain viable indefinitely, similar to frozen-stored mammalian spermatozoa. Also, frozen embryos exposed to low levels of radiation over an extended period of time have given no evidence of fetal abnormalities following their transfer to recipient females.

In farm animals, live offspring have been reported in the scientific literature from frozen-thawed embryos in sheep, goats, cattle and most recently the horse. Recently, Genetic Engineering News (1985) has reported that pregnancies have occurred from frozen swine embryos. However, live offspring from frozen swine embryos have not been reported in the scientific literature to date. The first frozen-thawed embryo transplant calf was born in the United States (California) in 1976. Since then, the numbers produced have increased to over 21,000 frozen-thawed calves per year in North America. Embryo freezing technology is now well developed for cattle and is on the increase for sheep, goat and horse embryos. In farm animals as a group, the expected pregnancy rates from frozen-thawed embryos range from 35 to 55%. Researchers propose that these percentages will likely increase in years to come as additional advancements are made in cryopreservation techniques.

The first frozen-thawed human in vitro fertilized baby born was reported by Alan Trounson's group in Australia in 1984. Since then, other IVF pregnancies have resulted from frozen-thawed human embryos. A popular press report recently indicated that over 150 human embryos are presently being stored in liquid nitrogen in Australia alone.

Projections.

The success rates following the transfer of frozen-thawed embryos of the mouse and the farm animals are presently greater than for humans. The farm animals embryos that exhibit the best viability following freezing are those frozen at the morula or blastocyst stage of development. To date, human embryos that have been frozen have been at a much earlier stage of development at the time of collection. In humans, the use of freezing technology has been hindered by ethical and legal restrictions rather than by the lack of scientific knowledge available on cryopreservation. Furthermore, research has not developed in human cryobiology due to the lack of availability of human embryos to conduct needed experiments. Freezing latter-stage human embryos would, in all probability, increase the post-thaw survival rates and increase chances for pregnancies.

The transfer of embryo freezing technologies developed in animals to humans would be very valuable. Developing successful methods freeze human embryos would be one of the most important aids to infertile couples in the years to come. Often the chances of an IVF pregnancy are markedly reduced when an embryo is placed back into the uterus that has been hyperstimulated during superovulatory treatment. If the embryos were frozen, they could be transferred non-surgically to the donor female several months later, when she has exhibited several normal

menstrual cycles. One or two frozen-thawed embryos could be transferred during successive cycles to increase the chances for a pregnancy. As a precautionary measure, career women could bank frozen embryos early in life to be used for transplantation later in their career. Since frozen mammalian embryos appear to remain viable for an indefinite period of time in cold storage, this approach to human infertility should not be overlooked coming years.

In the future, newer more efficient methods of freezing embryos in animals will become available to the human infertility clinics. The possibility of directly plunging the embryos into liquid nitrogen (Takeda and co-workers, 1985; Takahashi and Kanagawa, 1985; Williams and Johnson, 1986) would greatly simplify the freezing procedure and essentially make it available to every medical clinic who wishes the technology. A newer method to freezing embryos, called vitrification, is giving evidence of reduced cellular damage during the freezing process with mice embryos (Rall and Fahy, 1985), and some say this may be the approach to freezing mammalian embryos in the future.

Frozen Storage of Oocytes.

Progress.

Little success has been reported on freezing mammalian oocytes in the scientific literature. Furthermore, there is no evidence to date that live offspring have been produced from animal oocytes that had been previously frozen before fertilization. The majority of experiments on freezing ova has been with fresh mouse oocytes, and these were conducted to evaluate the physical and biochemical properties of the ovum, rather than to produce a zygote. Results thus far indicate that the oocytes

suggests that the zona pellucida may be more susceptible to structural damage during freezing, resulting in a greater number of bovine ova rupturing at post-thawing than would be expected from freezing day 6 or day 8 cattle embryos.

A major reason for not being able to produce offspring from frozen-thawed mammalian ova is that a proficient in vitro fertilization procedure is needed to generate the embryos for transfer to recipient females. Only recently have in vitro fertilization produced live offspring with cattle, sheep, goat and swine oocytes. A second reason for the lack of success to date is not having suitable in vitro culture systems for most animals to develop the fertilized ova from the 1 cell stage through to the early blastocyst stage, to obtain optimal transfer pregnancy rates.

Since offspring has recently resulted from frozen-thawed oocytes in humans, this is presently an area of increased interest. The human is the one species that has in place relatively efficient in vitro fertilization methodology, which usually results in a 50% or greater fertilization rate for normal oocytes. Also, there is less need for an in vitro culture system for human embryos.

Projections.

Laboratories outside the United States are presently conducting experiments on freezing human oocytes. Glycerol is the primary cryoprotectant being evaluated in these studies. Since human ova can be fertilized in vitro and developing embryos can be transferred at early developmental stages, it is feasible that the advancements in the next 5 years will likely be made with in vitro fertilization of previously frozen human oocytes. This could be a very important area of research

for human females with ovarian and uterine dominated fertility problems. Being able to store oocytes in liquid nitrogen would allow for controlled experiments to study human oocyte maturation — a subject area which little is known today.

Storage of frozen oocytes could be an alternative to permanent infertility for a female that had to have a bilateral ovariectomy early in life due to cystic ovarian disease. The oocytes could remain frozen until she married at which time the oocytes are thawed and fertilized in vitro with her husband's semen. For a woman with family inherited genetic defect, oocytes could be obtained from a frozen bank and fertilized with her husband's and the resulting embryo transferred to the wife to carry the pregnancy to term.

The possibility also exists that in the future oocytes could be collected from the female's ovary shortly after accidental death and the oocytes frozen in liquid nitrogen. The husband, in this case, could use his semen to fertilize the ovum in vitro and have the developing embryo be carried to term by a female member of the immediate family or another surrogate female.

The Potential for Frozen Storage of Spermatozoa.

Progress.

The potential for developing efficient procedures for freezing spermatozoa from laboratory and zoo animals has not been fully realized. Difficulty obtaining sperm cells from the male populations and the lack of potential economic importance of these animal species are often cited for the slow development of this sperm cell freezing methodology. In contrast, frozen cattle semen developed into a multi-million dollar

commercial business in the USA since 1949, primarily because of the addition of a cryoprotectant (glycerol) to the semen extender. Since this important finding, the technology for freezing bovine semen has now developed into standardized procedures throughout the world. Live offspring have also been produced from frozen semen in chickens, sheep, goats, horses and swine. Almost 20 years of research was put into freezing boar semen before the first live piglets were produced at Ed Grahman's laboratory at the University of Minnesota in 1971. Only minimal success has been reported for producing live offspring from zoo animals over the years. Recently, interest in sperm cell freezing has intensified because of current efforts to develop embryo transplant technology for rare and endangered species.

All indications thus far suggest the properly prepared mammalian spermatozoa frozen and stored in liquid nitrogen (-196°C) can withstand long term storage with little or no effect on post-thaw pregnancy rates. At least one case has been reported where a calf has been produced from frozen bull semen thawed over 30 years later.

In humans, the idea of freezing spermatozoa for later use dates back to the later 1800's, where vessels containing human sperm cells were placed in a snow drift for subsequent post-cooling observations. Correspondingly, the first pregnancy in women with frozen-thawed human semen was announced to over 30 years ago. By 1964, at least 24 children were born as the result of artificial insemination with frozen-thawed semen in the United States and Japan. Freezing technology continued to develop and by 1973 it was reported that nearly 600 births had been recorded from artificial insemination with low temperature-stored human sperm cells (Sherman, 1973). In 1984 it was estimated that more than

250,000 babies had been born in the USA as a result of frozen-thawed human semen. Today, successful insemination results with fresh and frozen semen are being reported from hospitals and fertility clinics worldwide.

One of the largest human sperm bank organizations in the United States (Georgia) now claims that it is sending out up to 100,000 inseminating units of human semen annually, and is expecting the market to increase in the future (The Observer, p. 3, February 7, 1983). With the human sperm banking steadily developing during the last 10 to 15 years, it now ranks second only to frozen bull semen as a commercial business. A recent estimate indicates that the success rate from frozen-thawed human semen ranges from 20 to 68%.

Projections.

In humans there appears to be a need for quality control measures and standards set up for packaging of human semen, much as was developed by commercial bull studs in North America some years ago. The bull studs have done this (certification program) among themselves, with their own structured organization. In the future, this approach should be recommended to the human semen banking industry.

During the next 10 years new developments in animal sperm cell physiology and biochemistry will continue at a slow but constant rate, and this technology will then be available to transfer to human medicine to improve male fertility levels. One method already under evaluation with human semen is the use of BSA (bovine serum albumin) to treat the sperm cells before insemination. Results reported in farm animals indicate that semen treated with BSA prior to insemination or freezing markedly improved progress motility rates. Preliminary findings from

these studies suggest that BSA may have a biochemical as well as a biological filtering effect on spermatozoa. Studies with animal spermatozoa suggest that the addition of prostaglandins to the semen extender prior to freezing or following freezing may enhance post-thaw viability of spermatozoa. Prostaglandins have been linked to some infertility problems related to low sperm cell concentrations in man. In the future, human semen may be treated with such chemical agents as chondroitin sulfate post-thawing to enhance the acrosome reaction (Lenz and co-workers, 1983) or heparin added to the semen after thawing to increase fertilization rates with artificial insemination.

Improved fertilization rates observed with heterospermic inseminations in animals may be re-evaluated with human semen, and this procedure may someday be used to improve the fertilization rates at human IVF clinics. Optimal post-thaw fertilization will be needed in the future to expand the human IVF procedures and to maximize use of frozen human semen in embryo transfer procedures.

Preliminary results in bovine studies indicate that when young bulls were actively immunized against testosterone there was an increase in testicular size and spermatogenic potential as the males reach maturity (Thompson and co-workers, 1973). Active immunization of human males to androgens may be one way of aiding a young males suffering from hypogonadism.

In the future, fresh and frozen thawed sperm cells will be used in humans to help reduce the possibility of spreading venereal disease and possibly the transmission various types of cancer-related DNA particles. Semen will also be evaluated and screened for AIDS before freezing and inseminations are performed. If advances are made in animal

fertilization with sperm microinjection experiments, this new fertilization technology should be transferable to human reproductive medicine without difficulty. During the next 5 to 10 years the possibility may also exist that human offspring could be born from in vitro fertilization using sperm microinjection of freeze-dried sperm DNA. If this was the case, a man killed in an accident could later father a child. The sperm cells would be collected at autopsy for freeze drying and microinjection could be performed on the wife's ovum at a later date.

In Vitro Culture Systems for Embryos.

Progress.

Once fertilization has occurred in vitro, the success rate following transfer to recipient females is quite low for most animals studied to date. The mouse and the pig, for example, have a little more tolerance to naturally fertilized early-stage transfers than some of the other mammals. For in vitro fertilized embryos of other animals, however, an in vitro culture system is needed to develop the embryos to a stage where optimal pregnancy rates occur following transfer.

Since adequate in vitro culture systems have been unavailable in the past, in vivo culture has been used to allow embryos to develop to later stages for subsequent transplantation to surrogate females. A classic example in this case is the use of the rabbit oviduct for short-term in vivo culture of farm animal embryos. Previous reports describe the transportation of embryos from one location to another while inside the oviducts of rabbits. Today, an agar embedding procedure developed by Willadsen (1979) is used to develop early-stage farm animal embryos in the ligated oviducts of diestrous or anestrous sheep before transfer

to recipient females. This in vivo culture technique is effective but considered too costly and time consuming to be used in most laboratories.

Although a multitude of different media have been evaluated for culture of mammalian embryos, only a few remain for modern-day culture of laboratory and farm animal embryos. Most researchers now agree that any of the fluid in vitro culture systems now available (e.g. TCM-199, Ham's F-10, CMRL-1066) will support later-stage embryo development for up to 36 or possibly 48 hours, after which transfer pregnancy rates decline markedly. Present media usually do not carry animal embryos through the embryo block stage of development. The "development block" is not evident in early-stage human embryos. Most commercial cattle embryo transfer companies now collect later-stage embryos, cool them to room temperature and then culturing them overnight, only as the need arises. It is now clear that improved embryo culture procedures need to be developed for mammalian embryos in the future.

In humans, oocytes are collected for in vitro fertilization usually by follicle puncture. After fertilization occurs, the embryos are culture in vitro until they are either 2-, 4- or 8-cells before they are transferred non-surgically into the uterus of a female. Usually due to laws implemented by society, human embryos are not cultured to later developmental stages, as with many of the mammalian species. The media most often used to support early cleaving human embryos in vitro includes Earle's, Ham's F-10, Hoppe and Pitts medium, usually with a protein source (albumin or serum) added. These medium preparations are relatively simple to prepare and are in wide use in human IVF clinics

today. Human embryos are usually cultured for no more than 2 or 3 days before their transfer using today's methodology.

Projections.

Developing in vitro culture systems for mammalian embryos will continue to be a priority for researchers in the years to come. With recent advances with IVF in farm animals and gene injection technology for ova, research on in vitro culture systems has intensified during the last 2 years. As IVF and embryo transfer becomes more widely accepted as treatments of human infertility, there will be a greater need to develop an in vitro embryo culture system for humans that will allow embryo development to continue from the 1-cell stage through to the blastocyst stage of development. At this time, the transfer of new animal embryo culture technology will be made to human reproductive medicine. There are many similarities between culture systems developed for animal embryos and those already being used at human IVF clinics.

At present, there is a different approach to culturing human embryos, and that is more towards an in vivo method, called "GIFT". GIFT (gamete intrafallopian transfer) is presently only available at several medical centers and is expected to become available to the others in the near future. For the GIFT procedure to be executed on infertile women, at least one healthy oviduct must be present in the female. With this technique, the ovum and sperm cells are carefully placed in the oviduct of the woman for fertilization to occur. This is different from allowing fertilization to occur in vitro with present IVF procedures. After in vivo fertilization occurs, the developing embryo travels through the oviduct and enters the uterus where implantation subsequently takes place or the embryos removed and placed in a

surrogate female. In a recent popular present article (USA Today), Ricardo Asch at the University of Texas Health Sciences Center in San Antonio has reported a 28% pregnancy rate on 150 women using the GIFT procedure, which was slightly higher than his pregnancy rate with a standard IVF procedure.

In the future, the fluid medium embryo culture systems will likely be all but replaced by monolayer fibroblast culture systems. Monolayer cultures will likely be derived from uterine endometrial cells, oviductal cells or possibly even tissues from other body tissues (e.g. hepatic cells). The monolayer culture system has been very successful for culturing farm animal embryos and should enhance the viability of human embryos placed in short-term culture. Monolayer systems appears to have a good future for culturing mammalian embryos.

Presently studies are being conducted with embryo and fetal growth factors being added to the culture system hoping to enhance in vitro culture viability. Socoseryl, an amino acid-based product from young bovine blood, appears to be embryotrophic in animal embryo culture studies to date (Viker and co-workers, 1984c). Epidermal, fibroblast and nerve cell growth factors are also being evaluated as a culture system supplement. Co-culture embryos with trophoblastic vesicles has recently given encouraging results with cattle embryo culture systems. There is some indication that these day 13 to day 15 trophoblastic vesicles produce an embryotrophic effect on day 6 to 8 bovine embryos. The transfer of these technologies to human medicine will likely occur during the next 3 to 5 years.

As procedures improve for culturing animal embryos, it is likely that more efficient method of evaluating embryo quality will also become

available. The use of various embryo staining procedures developed for animals to evaluate viability prior to transfer (e.g. FDA) will then be transferred over to human embryo technology. In the future, the possibility exists that embryo quality will be evaluated prior to embryo transfer via a computerized image analysis system (Youngs and co-workers, 1987). Preliminary results in this area appear very encouraging and should be transferable over to human reproductive medicine in the near future.

The Potential for Sexing Embryos.

Progress.

Sexing embryos by karyotyping techniques have been reported for mice and cattle. The most rapid of these methods allows for bovine embryos to be sexed by evaluating chromosome spreads starting 6 hours from the time of embryo collection. This approach to embryo sexing has not been readily accepted by commercial embryo transplant units because it is time consuming and tends to lower transfer pregnancy rates. Using karyotyping procedures, usually only 66 to 80% of the embryos can be sexed at the present level of technology.

Fluorescent antibody procedures for detecting H-Y antigen (male specific protein) on the surface of blastomeres of early-stage embryos was first reported in mice in the early 1980's. In the laboratory and farm animal species evaluated, H-Y histocompatibility antigens has been detected in embryos from the 2-cell stage through the blastocyst stage of development. Respectable results have been reported thus far in sexing mice and cattle embryos. With this noninvasive immunofluorescence assay, White and co-workers (1986a) have recently reported an

overall accuracy rate of 90% in sexing cattle embryos. Although limited data are available, using this monoclonal H-Y antibody sexing approach appears to have potential for sheep, swine and the horse embryos. Verification of this sexing procedure for other animals is presently underway.

The newest development in this area involves sexing embryos with a specific DNA probe. In a recent news release researchers in France announced that they had developed a DNA probe technique for sexing 7-day-old bovine embryos. Previous research efforts have given encouraging results using a DNA probe to sex mouse embryos. Granada Genetics, a privately-owned company in Texas, is also evaluating a specific DNA probe for sexing latter-stage bovine embryos. A spokesman for the company indicates that the results thus far are promising, however, the results from these trials have not been reported in the scientific literature.

Although sexing the fetus by karotyping embryonic cells (collected via amniocentesis) is a well established clinical procedure in humans, success sexing human embryos by karotyping for subsequent transfer has not been reported to date. Correspondingly, results from using H-Y antiserum and DNA probes to sex human embryos has not yet been reported in the scientific literature.

Projections.

In humans, it is likely that karotyping as a method for sexing embryos will not be extensively developed in the future, unless there is a general acceptance of embryo splitting methodology for human embryos. In this case, embryonic cells would be evaluated from half of the

bisected embryo while the remaining embryo half would be made ready for transfer.

There is no reason to believe that either the H-Y antibody or the DNA probe procedures could not be developed from existing animal technology for use in sexing human embryos. H-Y antigen has been detected on the surface of human somatic cells of the male and should therefore be present on the blastomeres of the human embryo. The drawback facing scientists in this area of research is the availability of human embryos for study. Using new antibody production technology, specific male DNA probes should become available in the near future for human embryos.

More recently, efforts to find a simple, noninvasive method of sexing mammalian embryos have been directed towards evaluating sex-specific gene products (e.g. small proteins, enzymes) produced by the embryo that could identify the Y chromosome in the nucleus of the embryonic cells. Preliminary results using mice and swine embryos are encouraging, however, more research is needed to verify these initial results.

Society has accepted amniocentesis and karyotyping as a method of determining the sex of the fetus while in utero. However, if the human embryo was sexed prior to transplantation, then a potential problem would exist if the potential parents preferred a baby of the opposite sex than the embryo. What to do with an embryo not accepted by the parents would then cause ethical problems to be faced by the medical profession. Possibly this embryo could be placed in an embryo adoption program for infertile couples.

Splitting Embryos to Produce Twins.

Progress.

The idea of bisecting embryos to produce genetically identical twin offspring is not a new one. Results were first produced from early-stage frog embryos that were bisected with a human body hair. Splitting laboratory animal embryos (mice) with a micromanipulator unit was first reported by Moustafa and Hahn (1978). In this experiment, morulae from laboratory mice were bisected and the halves transferred to recipient females resulting eight sets of genetically identical twin mice. Embryo splitting methodology for later-stage embryos (morulae and blastocysts) has now been developed for cattle, sheep, goats, swine and horses, in addition to the technology being available for laboratory animals. Some 50 or more commercial embryo transplant units worldwide are now using a simplified embryo splitting procedure for bovine embryos, and it is projected that this methodology will be used commercially on other farm animals and for zoo animal species in the near future.

To date embryo splitting has not been reported for human embryos in the scientific literature. This will likely be reported from laboratories outside the United States sometime during the next 2 years.

Projections.

Although embryo splitting procedures have not been reported for human embryos, it is likely that this technique will be as successful for human embryos as it has been with both large and small animals. As with embryo transfer in animals, splitting embryos of humans should increase the potential number of pregnancies per embryo collection. This would benefit infertile females, where scar tissue has built up

around the ovaries from repeated surgery or laparoscopic procedures, and for those females nearing the onset of menopause.

The possibility exists that the human embryo could be bisected and the embryonic cells from half of the embryo could be evaluated for genetic defects or used to determine the sex of the embryo via chromosomal analysis or antibody sexing techniques before transferring the remaining "half" embryo. These technologies are presently available for animal embryos and could easily be transferred to the human embryo model. In the future, embryo bisection may actually be executed by a computer-controlled automated embryo micromanipulation unit similar to the system developed for animal embryos by Ogawa and co-workers (1986) in Japan.

The Production of Chimeric Embryos.

Progress.

Microsurgical procedures for producing chimeric offspring from two or more embryos has been developed for mice, sheep, goats and cattle. Thus far the simplest approach to producing chimeric embryos is to aggregate intact or portions of morulae or early blastocysts (Rorie and co-workers, 1986). The newest approach is to use immunosurgery to remove the trophoblast cells and then to microinject the ICM cells from the embryo into the ICM cells of another intact blastocyst. Interspecies chimeras have been produced from sheep and goat embryos in England, and most recently by Gary Anderson's laboratory in California.

To date no reports are available in the scientific literature on the production of chimeric offspring from two or more human embryos.

Projections.

Although the procedure for producing human chimeric embryos has not been developed, there should be no reason why these embryos would not develop in a similar manner to those of other mammalian embryos reported to date. This procedure, however, has questionable ethical value for use in human infertility cases at the present time. This is an example where animal reproductive physiology technology is available for technology transfer, but will likely not be fully incorporated into human embryology studies in the years to come. One should not overlook the possibility of adding genetically-related trophoblastic cells from one embryo (those that form the placenta) to the trophoblastic cells of another embryo prior to the transfer of human embryos. This may aid infertile couples that face an in vivo conceptus rejection response during a natural pregnancy.

In the future, the developing intraspecies chimeric embryos for various mammals may have basic research value in studying growth potential, behavioral responses and immunological patterns in animals and man. The chimeric animal models developed for research should add new insight to the study of human embryology and physiology in the years to come. The interest in studying immunological development among competitive cell types in chimeric animals could be of great importance with the growing problem of AIDS in our human population.

Inter Cell Mass (ICM) Transfers Between Embryos.

Progress.

Some success has resulted following ICM transfers between embryos of the same and different species of animals. Unfortunately, the

results of many of these experiments have not been reported in the scientific literature, especially with several of the farm animal species. Live offspring (both sheep and goats) have resulted from ICM transfers between sheep and goats. Research underway at present in farm animals involves removing the trophoblast cells from the blastocyst-stage embryos by immunosurgery and transferring the ICM cells into another blastocyst (with ICM cells removed) using a fine glass micropipette attached to a micromanipulator unit.

At present, there are not reports of ICM transfers attempted on human embryos in the scientific literature.

Projections.

The primary use of this new embryo engineering will be in the area of zoo animal reproductive physiology. Being able to transfer the ICM cells (those which produce the fetus) to another embryo with the ICM cells removed would leave the transplanted ICM cells available to develop into a fetus within a placenta of a different embryonic origin (another embryo). With rare exotic mammals, the ICM cells from these embryos could be placed into the trophoblastic cells of embryos from a more abundant, related animal species. This approach would allow the endangered animal to serve as the offspring donor and the more abundant species to serve as recipient animals.

Although the ICM transfer technology is now being developed for animal embryos, this methodology will likely not be developed to the extent that embryo transfer technology will become during the next decade. The use of ICM transfers in humans would likely be in the cases of human infertility where there is a maternal-conceptus rejection response during a natural pregnancy. Although this type of rejection is

considered rare, some researchers propose this could be one of the causes of unexplained fertility in young married couples.

The Potential for Embryonic Cloning.

Progress.

Somatic cell nuclear cloning has produced offspring in amphibians (e.g. frogs), however, no verified experimental evidence has been presented in the scientific literature for adult cell cloning in the mammalian species. The first success with embryonic somatic cell cloning producing live offspring was reported with mice in the early 1980's. Research has continued in this area to verify these findings in mice.

Recently, the first farm animal embryonic cell clones (in sheep) were produced in Cambridge, England by Willadsen (1986). The lambs produced were the result of a blastomere from an 8-cell ovine embryo combined with a enucleated ovine "half" ovum. Recently, the newsline services (November, 1986) reported that Dr. Willadsen (visiting scientist at Granada Genetics in Marquez, Texas) had produced the first live nuclear transplant calves (embryonic somatic cell clones) in the world. In this case, a blastomere from an 8-cell bovine embryo was combined with unfertilized bovine ovum and transferred to recipient cattle. Other laboratories are performing similar procedures and unofficial reports indicate that pregnancies are underway in cattle and swine.

Although human cloning is a favorite topic of discussion in fiction stories, there is no scientific evidence reported that cloned offspring have been produced in man.

Projections.

The methodology for embryo nuclear transplantation is now being developed for laboratory and farm animals. A great deal of information

on the topic will likely appear in the public press in the months to come. Results thus far suggests that the nuclear material (DNA) of the 8-cell embryos evaluated has not differentiated and remains totipotent. This affords the theoretical opportunity to initially obtain eight cloned offspring from a single embryo. There is a real possibility that this nuclear transplantation procedure could be replicated on subsequent cloned embryos to increase the potential number of offspring that could be produced from an embryo. The use of the embryo cloning procedure now being developed for animals should also be applicable human embryos. In those human cases, where an aging female has only a slight chance of ova at collection, the opportunity for using nuclear transplantation technology on any embryo produced should greatly enhance her chance of obtaining a pregnancy.

Developing embryo-derived stem cell lines for subsequent use in nuclear transplantation is presently under investigation in several laboratories. Preliminary culture success with mouse stem cell cultures is encouraging at the present time. More research with animal models are needed in this area.

The idea of "cloning humans" from embryos will not readily be accepted by society and will likely be more difficult to overcome than developing the animal models for technology transfer to human reproductive medicine.

DNA (Gene) Injection for the Production of Transgenic Offspring.

Progress.

This area of research surfaced in 1981 with the birth of the first transgenic mice and has now become one of the most active areas of

embryo engineering research during the 1980's. This first approach to the producing transgenic laboratory animals used pronuclear injection of DNA material (gene from another animal) into recently sperm-activated mouse ova. Following fertilization, these DNA-injected embryos were then transplanted in recipient mice and live offspring were born. In this report, Wagner and co-workers (1981) presented results showing that the exogenous gene microinjected into the ova became incorporated into the mouse DNA and was expressed in the blood components of the mouse transplant offspring. Furthermore, there was some indication that the exogenous gene was then passed on to subsequent offspring, suggesting the possibility of germline transfer of foreign genetic material. During the last 5 years, other laboratories have microinjected recently activated mouse ova with different gene constructs and have now verified this new technology for producing transgenic mice.

More recently, researchers have been looking into ways of inserting man-made genes into activated ova and developing transgenic embryos in farm animals. One gene presently interesting researchers is the Growth Hormone (GH) gene, and the possibility of incorporating this genetic material into swine, sheep, goat and cattle ova. If this gene were to become incorporated into the nuclear DNA, the possibility exists that the GH gene construct could be activated to stimulate growth as the transplant offspring develop toward maturity. Successful incorporation and expression of the GH gene has already been reported in transplant mice (Palmiter and co-workers, 1982, 1983 and others).

The first transgenic farm animal offspring born were reported by Hammer and co-workers (1985). Pronuclei of activated ova of rabbits, pigs and sheep were microinjected with copies of the human GH gene which

had been fused to a mouse regulatory gene (MT-hGH). Of the transplant offspring born in this experiment, 1.3% of the lambs, 10.4% of the piglets and 12.8% of the newborn rabbits were detected as having some level of gene incorporation. Gene expression was detected in the rabbits and the pigs, but not detected in the gene-injected sheep offspring. Correspondingly, researchers at Texas A&M University produced six gene-injected transplant calves in 1985 (Gulf Coast Cattleman, January, 1986), however, only one of these calves has the possibility of being transgenic. Several research groups have gene-injected embryo transplant pregnancies now underway in swine and cattle in North America and are waiting for offspring to be born.

To date no reports have appeared in the scientific literature on the production of transgenic human embryos. Researchers in other countries are said to be presently investigating the possibility of transferring this transgenic animal technology over to human reproductive medicine.

Projections.

This area of research is one of the newest in the field of animal reproductive physiology. There appears to be very short lag-time between discovery and implementation, especially in the farm animal area. As the methodology for animal embryos develops, it should be readily available for technology transfer to human reproductive physiology. Although gene-injection technology offers a great deal of potential to many facets of human medicine, society will likely be the limiting factor to its growth and development in the years to come.

In the future, genetic defects and diseases of man will likely be the target of gene-incorporation technology. There are estimated to be

some 3,000 human disorders which have some relation to genetics that have now been identified in man. For the 200 or more human genetic disorders which are thought to result from a single gene effect, this new genetic technology will offer the opportunity of changing these maladies in transplant offspring, and possibly in subsequent family generations. The genetic defects or diseases that have already been identified as single gene effects, will likely be among the first to be considered for gene-incorporation technology. Some of these genetic disorders include adenosine deaminase defect, muscular dystrophy, cystic fibrosis, sickle cell anemia, hemophilia, Huntington's chorea, and possibly Alzheimers. By the year 2000, it has been estimated that most of the genes responsible for major birth defects that cripple some 15 million US residents will be mapped, opening the possibility for treatment with gene-incorporation technology.

Studies are now being conducted using non-replicating viral vectors to transport genes into the embryonic cells of later-stage animal embryos. Preliminary in vitro results using this approach to gene-incorporation into mouse embryos has been encouraging. This basic methodology is now being attempted with farm animal embryos.

If gene incorporation technology is developed for humans, the possibility exists that a woman could enter the outpatient clinic, have her day 5 to day 8 embryo collected non-surgically, the genetic material placed into the embryo with a non-replicating viral vector and the embryo non-surgically replaced into her uterus before she leaves the outpatient clinic.

As gene-incorporation technology develops in the years to come there appears to be a great deal of potential for human medicine.

Other Reproductive Technologies that may Be Applicable to Humans.

The following is a listing of various reproductive technologies that may in the future have some application to human reproductive physiology. Many of these relatively new items did not fit under the topic areas of the project outline for this OTA manuscript, therefore they were included in this listing:

1. Sweat and saliva components to evaluate human fertility
2. Computerized system (Micron Medical Inc., Boise, Idaho) that predicts and confirms ovulation in women.
3. Antibody to the zona pellucida (embryo) administered to women to inhibit fertilization (Tsunada and co-workers, 1981).
4. Antibody to human chorionic gonadotropin (HCG) administered as a human fertility control method.
5. Administering glycosaminoglycans to females to improve fertilization rates.
6. New slow-release steroid implants used to allow a bilaterally ovariectomized female to carry an embryo transplant to term.
7. Monitoring short-term prostaglandin production by the embryo to evaluate potential viability prior to freezing or transfer.
8. Using standard refrigeration for short-term storage of human embryos prior to transfer (Pool and co-workers, 1986).
9. New immunological methods for early human pregnancy detection (e.g. alpha fetal protein).
10. Early Pregnancy Factor (EPF) to aid in the study of infertility in women.
11. Fetal-cell surgery procedures to treat human disorders (Time, p. 62, January 12, 1987).
12. The use of human embryonic cells in the search for cancer treatment.
13. Exogenous gene therapy to fight human diseases in children and adults.
14. The new technology of "DNA finger printing" to help solve rape cases.

PART III

Summary

of the

Potential Application of Animal Reproductive

Technologies

to Humans

(Condensed Format)

Part III.

TABLE 8. RESEARCH POTENTIAL FOR REPRODUCTIVE TECHNOLOGIES
DURING THE NEXT DECADE¹

Technology x Mammalian Group	Laboratory Animals	Farm Animals	Humans
1. Non-surgical embryo transfer methodology	5	6	9*
2. Gonadal tissue transplantation	3	6	8*
3. Superovulation methodology	6	9*	8
4. Maturation of oocytes <u>in vitro</u> (live young)	5	8*	8*
5. Methods for sexing sperm cells	4	6*	6*
6. Treatment of spermatozoa to increase fertility	5	9*	8
7. <u>In vitro</u> fertilization methodology	5	7	9*
8. Sperm cell microinjection for fertilization	6	7*	7*
9. Methods for freezing embryos	7	9*	8
10. Methods for freezing oocytes	6*	5	4
11. Methods for freezing and storage of sperm cells	5	9*	8
12. <u>In vitro</u> culture system methodology	7	8*	7
13. Methods for sexing embryos	7	8*	8*
14. Splitting embryo procedures	8	10*	9
15. Production of chimeric embryos	7	9*	2
16. Procedures developed for inner cell mass transfers	6	8*	4
17. Methods for embryonic cloning developed	6	8*	5
18. Methodology for gene insertion	9	8*	5
Composite Score	= 107.0	140.0	123.0
Mean Technology x Group	= 5.9	7.8	6.8
Number of Top Rankings	= 1	14	7
Number of Bottom Rankings	= 13	0	6

¹Progress scores = 10 highest to 1 lowest based on information available.

Part III.

TABLE 9. POTENTIAL BENEFIT TO ADVANCEMENT OF REPRODUCTIVE PHYSIOLOGY DURING THE NEXT DECADE¹

Technology x Mammalian Group	Laboratory Animals	Farm Animals	Humans
1. Non-surgical embryo transfer methodology	3	7	8*
2. Gonadal tissue transplantation	2	8	9*
3. Superovulation methodology	4	7	8*
4. Maturation of oocytes <u>in vitro</u> (live young)	6	9*	8
5. Methods for sexing sperm cells	2	9*	7
6. Treatment of spermatozoa to increase fertility	2	6*	6*
7. <u>In vitro</u> fertilization methodology	4	8*	8*
8. Sperm cell microinjection for fertilization	2	7*	6
9. Methods for freezing embryos	4	9*	9*
10. Methods for freezing oocytes	4	8*	6
11. Methods for freezing and storage of sperm cells	3	7	8*
12. <u>In vitro</u> culture system methodology	8	9*	7
13. Methods for sexing embryos	4	8*	8*
14. Splitting embryo procedures	2	8*	7
15. Production of chimeric embryos	5	6*	1
16. Procedures developed for inner cell mass transfers	4	8*	4
17. Methods for embryonic cloning developed	7	9*	5
18. Methodology for gene insertion	6	8*	8*
Composite Score	= 72.0	141.0	123.0
Mean Technology x Group	= 4.0	7.8	6.8
Number of Top Rankings	= 0	14	9
Number of Bottom Rankings	= 13	0	3

¹Progress scores = 10 highest to 1 lowest based on information available.

Part III.

TABLE 10. BASED ON OUR SOCIETY TODAY, EXPECTED PROGRESS IN
REPRODUCTIVE TECHNOLOGIES DURING THE NEXT DECADE¹

Technology x Mammalian Group	Laboratory Animals	Farm Animals	Humans
1. Non-surgical embryo transfer methodology	3	4	6*
2. Gonadal tissue transplantation	2	4*	3
3. Superovulation methodology	4	7*	6
4. Maturation of oocytes <u>in vitro</u> (live young)	4	5	7*
5. Methods for sexing sperm cells	3	5*	5*
6. Treatment of spermatozoa to increase fertility	4	5*	3
7. <u>In vitro</u> fertilization methodology	4	6	8*
8. Sperm cell microinjection for fertilization	4	5*	4
9. Methods for freezing embryos	6	8*	7
10. Methods for freezing oocytes	5*	4	3
11. Methods for freezing and storage of sperm cells	4	8*	6
12. <u>In vitro</u> culture system methodology	7*	7*	6
13. Methods for sexing embryos	7	8*	6
14. Splitting embryo procedures	7	9*	6
15. Production of chimeric embryos	6	8*	1
16. Procedures developed for inner cell mass transfers	4	7*	2
17. Methods for embryonic cloning developed	5	8*	2
18. Methodology for gene insertion	7*	7*	4
Composite Score	= 86.0	115.0	85.0
Mean Technology x Group	= 4.8	6.4	4.7
Number of Top Rankings	= 3	14	4
Number of Bottom Rankings	= 9	0	9

¹Progress scores = 10 highest to 1 lowest based on information available.

Part III.

SUMMARY

TABLE 11. SPECIES X POTENTIAL REPRODUCTIVE TECHNOLOGY MATRIX SUMMARY

Technology x Mammalian Group	Laboratory Animals	Farm Animals	Humans	OTA Ranking ¹
1. Non-surgical embryo transfer methodology	Low	Medium	High	3
2. Gonadal tissue transplantation	Low	Medium	Medium	2
3. Superovulation methodology	Medium	High	High	3
4. Maturation of oocytes <u>in vitro</u> (live young)	Medium	Medium	High	3
5. Methods for sexing sperm cells	Low	Medium	Medium	2
6. Treatment of spermatozoa to increase fertility	Medium	High	Low	1
7. <u>In vitro</u> fertilization methodology	Medium	Medium	High	3
8. Sperm cell microinjection for fertilization	Medium	Medium	Medium	2
9. Methods for freezing embryos	High	High	High	3
10. Methods for freezing oocytes	Medium	Medium	Low	3
11. Methods for freezing and storage of sperm cells	Medium	High	Medium	3
12. <u>In vitro</u> culture system methodology	High	High	Medium	3
13. Methods for sexing embryos	High	High	High	2
14. Splitting embryo procedures	High	High	Medium	1
15. Production of chimeric embryos	Medium	High	Low	1
16. Procedures developed for inner cell mass transfers	Medium	High	Low	1
17. Methods for embryonic cloning developed	Medium	High	Low	1
18. Methodology for gene insertion	High	High	Medium	2

¹Progress scores = the highest = High to lowest = Low based on information available.

²OTA priority rankings = 3 highest, 2 = moderate and 1 = lowest.

Part III.

SUMMARY

TABLE 12. TIME-SCALE X TECHNOLOGY MATRIX FOR SIGNIFICANT DEVELOPMENTS

Technology x Mammalian Group	Laboratory Animals	Farm Animals	Humans	OTA Ranking ¹
1. Non-surgical embryo transfer methodology	5-10	1-5	1-5	3
2. Gonadal tissue transplantation	3-7	1-5	5-7	2
3. Superovulation methodology	1-5	1-5	1-5	3
4. Maturation of oocytes <u>in vitro</u> (live young)	5-7	5-7	1-5	3
5. Methods for sexing sperm cells	5-10	5-10	5-10	3
6. Treatment of spermatozoa to increase fertility	1-5	1-5	1-10	1
7. <u>In vitro</u> fertilization methodology	1-10	5-7	1-5	3
8. Sperm cell microinjection for fertilization	1-5	1-5	5-10	2
9. Methods for freezing embryos	1-5	1-5	3-7	3
10. Methods for freezing oocytes	1-5	1-5	3-7	3
11. Methods for freezing and storage of sperm cells	1-10	1-5	1-5	3
12. <u>In vitro</u> culture system methodology	1-5	1-5	3-7	3
13. Methods for sexing embryos	1-3	1-3	1-5	2
14. Splitting embryo procedures	1-3	1-3	3-7	1
15. Production of chimeric embryos	1-3	1-3	5-10	1
16. Procedures developed for inner cell mass transfers	1-3	1-3	7-10	1
17. Methods for embryonic cloning developed	1-3	1-3	7-10	1
18. Methodology for gene insertion	1-3	1-3	1-5	2

¹Progress scores = give in range of year for significant development based on information available.

²OTA priority rankings = 3.highest, 2 = moderate and 1 = lowest.

Part III.

Concluding Remarks.

Progress.

Textbooks tell us that the human body is a complex organism composed of some 60 trillion cells. Each human somatic cell has 46 chromosomes comprised of DNA which is thought to be made up of over 100,000 individual genes. Although molecular biologists have made great strides on the study of the DNA molecule with computer models in recent years, only some 800 of these genes have been mapped out. One recent estimate indicates that it will take a great deal of effort to complete the human gene mapping somatic cells by the year 2,000. Man prides himself with a vast knowledge of bodily function and recent progress made in medical research. Through these efforts human life expectancy has increased from 47 years in the early 1900's to almost 75 years in the 1980's. The progress made in recent years by scientists and practitioners certainly can not be denied.

Unfortunately, progress in developing technologies in human reproductive physiology has been far less than in other areas of biological science. The reason for this is not completely clear, but it appears that society's fears and taboos take longer to change and evolve than it takes science to develop the technology. This is still the case when working with human reproduction and infertility. Until this philosophy changes to some degree in our society, technology transfer of reproductive techniques and "know how" from animals to man will be hindered. It is interesting to note that marked changes may be occurring in the public's awareness to human reproduction problems with our nation's

concern with AIDS. Public awareness and education usually results in public support of worthwhile technology.

In evaluating the progress made thus far in developing reproductive technologies, the authors were somewhat surprised to find that potential technology advancements in humans were near, but philosophical, religious and ethical problems seemed to be blocking further development of these technologies. In spite of the "braking" effect of society, in some areas human reproductive physiology has had more success in the areas of freezing spermatozoa, artificial insemination, in vitro culture systems for oocytes, maturation of oocytes, in vitro fertilization and semen sexing than virtually all of the laboratory and farm animals evaluated to date. The possible exception in several of these categories would have to freezing spermatozoa and artificial insemination in the cow.

In the Future.

If human embryos were available for study, man would likely make significant advancement in other related areas of reproductive technology outlined in this manuscript. The primary reason why technological advances have been made with cattle — they are an economically important species. Also, it should not be overlooked that the cattle industry requested these reproductive technologies from the scientific community. These authors predict that reproductive technologies will also advance humans, as they have with laboratory and farm animals, if society wants these technologies, and is willing to support research efforts to meet this end.

References

- Acosta, R.A., Randel, R.D., and Ocanto, D.J., "Auto and Heterotransplantation of the Ovary to the Uterus or Parotid Region in Beef Cows and Prepuberal Heifers," *Theriogenology* 17:459-468, 1982.
- Adams, C.E., (Unpublished Data), 1975.
- Adams, C.E., Moor, R.M., and Rowson, L.E.A., "Survival of Cow and Sheep Eggs in the Rabbit Oviduct," *Proc., Vith Intl. Congr. Anim. Reprod. Artif. Insem. (Paris)* 1:573, 1968.
- Allen, W.R., Stewart, F., Trounson, A.D., Tischener, M., and Bielanski, W., "Viability of Horse Embryos after Storage and Long-Distance Transport in the Rabbit", *J. Reprod. Fertil.* 47:387-390, 1976.
- Allen, W.R., and Rowson, L.E.A. "Transfer of Ova Between Horses and donkeys." *Proc., VIIth Intl. Congr. Anim. Reprod. Artif. Insem., (Munich)* 1:483-488, 1972.
- Allen, R.L., and Wright, Jr., R.W., "Observations on the In Vitro Formation, Development and Differentiation of Trilaminar Vesicles Formed from Enzymatically Dispersed Porcine Blastocyst," *Theriogenology* 23: 333-345, 1985.
- Anderson, L.L., Schultz, J.R., and Melampy, R.M., *In: Gonadotropins: Their Chemical and Biological Properties and Secretary Control*, H. H. Cole (ed.) (San Francisco: W. H. Freeman and Co., 1964), pp. 171-219.
- Archbald, L.F., Baker, B.A., Clooney, L.L., and Godke, R.A., "A surgical method for collecting canine embryos after induction of estrus and ovulation with exogenous gonadotropins." *Vet. Med. Small Anim. Clin.* 75:228-238, 1980.
- Archbald, L.F., and Godke, R.A. "What are the risks for transmission of disease with embryo transfer?" *Proc. Conf. Artif. Insem. Embryo Transf. in Beef Cattle, NAAB (January 14), Denver, CO.* pp. 41-46, 1984.
- Averill, R.L.W., Adams, C.E., and Rowson, L.E.A., "Transfer of Mammalian Ova Between Species", *Nature (London)* 176-167, 1955.
- Baird, D.T., Goding, J.R., Ichidawa, Y., and McCracken, J.A., "The Secretion of Steroids from the Autotransplanted Ovary in the Ewe Spontaneously and in Response to Systemic Gonadotropin," *J. Endocrinol.* 42:283, 1968.
- Baker, B.A., Guillory, B.M., Abdulla, G.J., and Godke, R.A. "A technique for non-surgical collection of single ovulated bovine embryos." *Proc. Louisiana Acad. Sci.* 42:76, 1979.
- Baker, R.D., Eberhard, B.E., Leffel, R.E., Rohde, R.F., and Henschen, T.J. "Pregnancy rates following surgical transfer of bovine demi-embryos." *Proc. Xth Intl. Congr. Anim. Reprod. Artif. Insem. (June 10-14), University of Illinois, Urbana* 2:220-222, 1984.

- Baker, R.D., and Shea, B.F. "Commercial Splitting of Bovine Embryos." *Theriogenology* 23:3-12, 1985.
- Ball, G.D., Leibfried, M.L., Lenz, R.W., Ax, R.L., and First, N.L., "Maturation and Fertilization of Bovine Oocytes In Vitro". *Theriogenology* 19:112, 1983.
- Bank, H. and Maurer, R.R., "Survival of Frozen Rabbit Embryos", *Exp. Cell. Res.* 89:188-196, 1974.
- Bavister, B.D., Collins, K., and Eisele, S., "Non-Surgical Embryo Transfer in the Rhesus Monkey", *Theriogenology* 23:177, 1985.
- Beal, W.E., White, L.M., and Garner, D.L., "Sex Ratio After Insemination of Bovine Spermatozoa Isolated Using a Bovine Serum Albumin Gradient," *J. Anim. Sci.* 58:1436-1438, 1984.
- Beatty, R.A., "Separation of X and Y Spermatozoa," *Bibliog. Reprod.* 23(1-6):120-129, 1974.
- Beck, W.W., and Silverstein, I., "Variable Motility Recovery of Spermatozoa Following Freezing Preservation", *Fertil. Steril.* 26:863, 1975.
- Beernink, F.J., and Ericsson, R.J., "Male Sex Preselection Through Sperm Isolation", *Fertil. Steril.* 38:493, 1982
- Betteridge, K.J., "Homotransplantation of Ovaries with Vascular Anastomoses in Rabbit: Response of Transplants to Human Chorionic Gonadotropin," *J. Endocrinol.* 47:451, 1970.
- Betteridge, K.J. "Embryo Transfer in Farm Animals: A Review of Techniques and Applications." Canadian Department of Agriculture, Monograph No. 16, 1977.
- Betteridge, K.J., "A Historical Look at Embryo Transfer", *J. Reprod. Fertil.* 62:1-13, 1981.
- Beyth, Y., and Folishuk, W.Z., "Ovarian Implantation into the Uterus (Estes Operation): Clinical and Experimental Evaluation," *Fertil. Steril.* 32:657, 1979.
- Bhattacharya, B.C., Bangham, A.D., Cro, R.J., Keynes, R.D., and Rowson, L.E.A., "An Attempt to Predetermine the Sex of Calves by Artificial Insemination with Spermatozoa Separated by Sedimentation," *Nature (London)* 211:863, 1966.
- Billingham, R.E., and Parkes, A.S., "Studies on the Survival of Homografts of Skin and Ovarian Tissue in Rats," *Proc. Royal Soc. B (London)* 143:55, 1955.
- Bilton, R.J., and Moore, N.M. "In vitro culture, storage and transfer of goat embryos." *Australian J. Biol. Sci.* 29:125, 1976.

- Binns, R.M., Harrison, F.A., Heap, R.B., and Linzell, "Reproductive Behavior in the Sheep and Pig after Transplantation of the Ovary," J. Reprod. Fertil. 20:356, 1969.
- Bines, J.A., Hart, I.C., and Morant, S.V. "Endocrine Control of Energy Metabolism in the Cow: The Effect on Milk Yield and Levels of Some Blood Constituents with Injection of Growth Hormone and Growth Hormone Fragments." British J. Nutr. 43:179-188, 1980
- Blaha, G.C., "Effect of Age on the Donor and Recipient on the Development of Transferred Golden Hamster Ova", Anat. Rec. 150:413, 1964.
- Blakewood, E.G. and Godke, R.A., "The Potential for Frozen Gametes, Artificial Insemination and Embryo Transfer for Captive Breeding of Mammals", Proc. Amer. Assoc. Zool. Parks & Aquariums, pp. 68-69, 1986.
- Blaxter, J.H.S., "Sperm Storage and Crossfertilization of Spring and Autumn Spawning Herring", Nature (London) 172:1189-1190, 1953.
- Boland, M.P., Nancarrow, C.D., Hoskinson, R.M., Murray, J.D., Scaramuzzi, R.J., Radford, H.M., Avenell, J.A., and Bindon, B.M., "Superovulatory Response in Cows Following Immunization Against Testosterone and Treatment with Bovine Follicular Fluid and PMSG", Theriogenology 23:185, 1985.
- Brackett, B.G., Extracorporal Fertilization of Mammalian Ova, In: Gynecologic Endocrinology, J. J. Gold (ed.) 2nd Ed., (New York: Harper and Row, 1975), pp. 621-644.
- Brackett, B.G., "In Vitro Fertilization and Its Assessment with Embryo Culture," In: H. Hawk (ed.) BARC Symposium No. 3, Beltsville, MD, pp. 171-193, 1981.
- Brackett, B.G. Bousquet, D., Boice, M.L., Donawick, W.J., Evans, J.F., and Dressel, M.A., "Normal Development Following In Vitro Fertilization in the Cow," Biol. Reprod. (Suppl. 1) 24:173, 109A, 1981.
- Brackett, B.G., Keefer, C.L., Troop, C.G., Donawick, W.J., and Bennett, K.A., "Bovine Twins Resulting from In Vitro Fertilization", Theriogenology 21:224, 1984.
- Bradford, G.E., and Kennedy, B.W., "Genetic Aspects of Embryo Transfer. Theriogenology 13:13-26, 1980.
- Brand, A., Drost, M., Aarts, M.H., and Gunnink, J.W. "Device for Nonsurgical Transfer of Bovine Embryos and its effect on uterine contamination." Theriogenology 6:509-514, 1976.
- Brem, G., Brenig, B., Goodman, H.M., Seldon, R.C., Graf, F., Kruff, B. Springman, K., Meyer, J., Winnacker, E.L., and Kraublich, H., "Production of Transgenic Mice, Rabbits and Pigs by Microinjection", Theriogenology 25:143, 1986.

Brem, G., Kruff, B., Szilvassy, B., and Tenhumberg, H., "Identical Simmental Twins Through Microsurgery of Embryos", *Theriogenology* 21:225, 1984.

Brem, G., Tenhumberg, H., and Kraublich, H. "Chimerism in Cattle Through Microsurgical Aggregation of Morulae", *Theriogenology* 22:609-613, 1984.

Brem, G., Tenhumberg, H., Kruff, B., and Kraublich, H., "Production of Cattle Chimerae Through Embryo Microsurgery", *Theriogenology* 23:182, 1985.

Bromhall, J.D., "Nuclear Transplantation in the Rabbit Egg", *Nature (London)* 258:719-721, 1975.

Brown, J.E., and Harris, Jr., G.C., "The Influence of Glycerol Equilibration Time on the Metabolism, Motility and Fertility of Frozen Chicken Spermatozoa", *Poultry Sci.* 42:377-380, 1963.

Buell, J.R., "Method for Freezing Stallion Semen and Tests of Its Fertility", *Vet. Rec.* 75:900-902, 1963.

Bunge, R.G., Keettel, W.C., and Sherman, J.K., "Clinical Use of Frozen Semen", *Fertil. Steril.* 5:520-529, 1954.

Bustillo, M., and Buster, J.E., "Nonsurgical Ovum Transfer: The Harbor UCLA Experience", *J. IVF Embryo Transf.* (Suppl. 1) 3:84a, 1986.

Butler, J.E., Anderson, G.B., BonDurant, R.H., and R.L. Pashen, "Production of Ovine Chimeras", *Theriogenology* 23:183, 1985.

Byrd, W. and Wolf, D.P., "Acrosomal Status in Fresh and Capacitated Human Ejaculated Sperm", *Biol. Reprod.* 34:859-869, 1986.

Camous, S., Heyman, Y., and Menezo, Y., "In Vitro Culture of Early Bovine Embryos with Trophoblastic Vesicles: Cleavage Through the Block Stage, Followed by Preganancy after Transfer," *Theriogenology* 21:226, 1984a.

Camous, S., Heyman, Y., Meziou, W., and Menezo, Y., "Cleavage Beyond the Block Stage and Survival after Transfer of Early Bovine Embryos Cultured with Trophoblastic Vesicles," *J. Reprod. Fertil.* 72:479-485, 1984b.

Chang, M.C. "Fertilization of Rabbit Ova In Vitro," *Nature (London)* 184:466-467, 1959.

Chang, M.C. "Artificial insemination of Snowshoe hares (Lepus americanus) and the transfer of their fertilized eggs to the rabbit (Oryctolagus cuniculus)", *J. Reprod. Fertil.* 10:447-449, 1965.

Chang, M.C., "In Vitro Fertilization in Mammal Eggs," *J. Anim. Sci.* (Suppl. 1) 27:15, 1968.

Chang, M.C. "Reciprocal Insemination and Egg Transfer Between Ferrets and Mink", *J. Exper. Zool.* 168:49-60, 1968.

Cheng, W.T.K., Polge, C., and Moor, R.M. "In Vitro Fertilization of Pig and Sheep Oocytes," Handout Supplement at the International Embryo Transfer Society Meeting, Colorado Springs, Colorado, (January), pp. 1-4, 1986.

Cho, F., Honjo, S., and Makita, T., "Fertility of Frozen-Preserved Spermatozoa of Cynomolgus Monkeys", Contemporary Primatology, Intl. Congr. Primatol. 5:125-133, 1974.

Clayton, O., and Kuehl, T.J., "The First In Vitro Fertilization and Embryo Transfer in Nonhuman Primates", Theriogenology 21:228, 1984.

Cole, R.J., and Paul, J., "Properties of Cultured Preimplantation Mouse and Rabbit Embryos, and Cell Strains Derived from Them," In: Preimplantation Stages of Pregnancy, Ciba Foundation Symposium, G. E. W. Wolstenholme and M. O'Connor (ed.) (Boston: Little, Brown and CO., 1965), pp. 82-112.

Coonrod, S.A., Bowen, J., and Kraemer, D.C., "Non-surgical Collection of Ovine Embryos," Proc. Vth Annual Conv. Amer. Embryo Transf. Assoc. (October 18-21) Fort Worth, TX, pp. 83-87, 1986.

Cross, P.C., and Brinster, R.L., "In Vitro Development of Mouse Oocytes." Biol. Reprod. 3:298-307, 1970.

Croxatto, H.B., Fuenteala, B., Diaz, S.; Pastene, L., Tutum, H.J., "A Simple Nonsurgical Technique to Obtain Unimplanted Eggs from Human Uteri", J. Obstet. Gynec. 112:622-688, 1972.

Danner, M.L., Oxender, W.D., Fogwell, R.L., and Douglas, R.H., "Use of An Equine Pituitary Extract With and Without HCG to Superovulate Cows", Theriogenology 11:96, 1979.

Davis, G.H., Montegomery, G.W., Allison, A.J., Kelly, R.W., and Bray, A.R., "Segregation of a Major Gene Influencing Fecundity in Progeny of Booroola Sheep", New Zealand J. Agric. Res. 25:525-529, 1982.

Dickerson, G.E., and Willham, R.L., "Quantitative genetic engineering of more efficient animal production", J. Anim. Sci. 57:248-264, 1983.

Desjardins, R.M., King, W.A., and Bousquet, D., "In Vitro Maturation of Horse Oocytes," Theriogenology 23:187, 1985.

Diedrich, K., Al-Hasani, S., van der Ven, H., and Krebs, D., "Successful In Vitro Fertilization of Frozen-Thawed Rabbit and Human Oocytes", J. IVF Embryo Transf. 3:65-66, 1986.

Dixon, K.E., Songy, Jr., E., Thrasher, D.M., and Kreider, J.L., "Effect of Bovine Serum Albumin on the Isolation of Boar Spermatozoa and Their Fertility," Theriogenology 13:437-444, 1980.

Dmowski, W.R., Gaynor, L., Rao, R., Lawrence, M., and Schommegna, A., "Use of Albumin Gradients for X- and Y-Sperm Separation and Clinical Experience with Male Sex Preselection," Fertil. Steril. 31:52-57, 1979.

- Donahue, R.P., "The Relation of Oocyte Maturation of Ovulation in Mammals," In: Oogenesis, J. D. Biggers and A. W. Schuetz (ed.) (Baltimore: University Park Press, 1972), pp. 413-438.
- Dowling, D.F., "Problems of Transplantation of Fertilized Ova", J. Agric. Sci. 39:374-396, 1949.
- Drost, M., Brand, A., and Aarts, M.H. "Device for Non-Surgical Recovery of Bovine Embryos", Theriogenology 6:503-507, 1976.
- Dubose, K.R., Kreider, J.L., Dixon, K.E., and Godke, R.A. "Non-surgical collection procedure for cycling mares." Proc. Louisiana Acad. Sci. 43:175, 1980.
- Durrant, B., and Benirschke, K., "Embryo Transplantation in Exotic Animals", Theriogenology 15:77-83, 1981.
- Edwards, R.G., Purdy, J.M., Steptoe, P.C., and Walter, D.E., "The Growth of Human Preimplantation Embryos In Vitro", Amer. J. Obstet. Gynecol. 141:408-416, 1981.
- Eichwald, E.J., and Simser, C.R., Untitled Paper, Transplantation 2:148, 1955.
- Elsden, R.P., Hasler, J.F., and Seidel, Jr., G.E., "Non-surgical Recovery of Bovine Eggs", Theriogenology 6:523-532, 1976.
- Emmens, C.W., and Blackshaw, A.W., "The Fertility of Frozen Ram and Bull Semen", Australian Vet. J. 31:76-79, 1955.
- Ericsson, R.J., "Isolation and Storage of Progressively Motile Human Sperm," Andrologia 9:111-114, 1977.
- Ericsson, R.J., Cassou, B., and Dapremant, G., "Isolation of Progressively Motile Mammalian Sperm: To Select for Y Sperm or Improve Fertility," Proceedings, IX Intl. Congr. Anim. Reprod. Artif. Insem. III:286, 1980.
- Ericsson, R.J., and Glass, R.H., "Isolation of Progressively Motile Sperm from Infertile Men", Fertil. Steril. 28:328, 1977.
- Ericsson, R.J., Langevin, C.N., and Nishino, M., "Isolation of Fractions Rich in Human Y Sperm," Nature (London) 246:421-425, 1973.
- Evans, J.M., Douglas, T.A., and Renton, J.P., "A Attempt to Separate Fractions Rich in Human Y Sperm," Nature 253:352-354, 1975.
- Eyestone, W.H., Vignieri, J., and First, N.L., "Co-culture of Early Bovine Embryos With Oviductal Epithelium", Theriogenology 27:228, 1987.
- Fabricant, J.D., Nuti, L.C., Minhas, B.S., Baker, W.C., Capehart, J.S., Marrack, P., Chalmers, Jr., J.H., Bradbury, M.W., and Womack, J.E., "Gene Transfer in Goats", Theriogenology 27:229, 1987.

- Faust, A.M., Kreider, J.L., Ericsson, R.J., Goodeaux, S.D., and Godke, R.A., "Isolation of Progressively Motile Spermatozoa from Bull Semen," *J. Anim. Sci.* 43:283, 1976.
- Fehilly, C.B., Willadsen, S.M., and Tucker, E.M., "Experimental Chimerism in Sheep", *J. Reprod. Fertil.* 70:347-351, 1984.
- Fehilly, C.B., Willadsen, S.M., and Tucker, E.M., "Interspecific Chimaerism Between Sheep and Goat", *Nature (London)* 307:634-636, 1984.
- Feichtinger, W., "Freezing Human Oocytes Using Ultra Rapid Techniques", *J. IVF Embryo Transf.* 3:68, 1986.
- Fekete, E., and Little, C.C., "Observations on the Mammary Tumor Incidence of Mice Born from Transferred Ova", *Cancer Res.* 2:525-530, 1942.
- First, N.L., Crister, E.S., and Robl, J.M., "Bovine Embryo: Developmental, Cloning, Sexing and Transfer of Genes," In: *Immunological Approach to Contraception and Promotion of Fertility*, G. P. Talwer (ed.) (New York: Plenum Press, 1986), pp. 375-392.
- Foot, R.H., and Onuma, H., "Superovulation, Ovum Culture and Transfer - A Review," *J. Dairy Sci.* 53:1681-1692, 1970.
- Fowler, R.E., and Edwards, R.G., "Induction of Superovulation and Pregnancy in Mature Mice by Gonadotrophins," *J. Endocrinol.* 15:374, 1957.
- Frazer, A.F., "A Technique for Freezing Goat Semen and Results of a Small Breeding Trial", *Canadian Vet. J.* 3:133-144, 1962.
- Friberg, J., and Gemzell, C., "Inseminations of Human Sperm After Freezing in Liquid Nitrogen Vapors with Glycerol or Glycerol-Egg Yolk-Citrate as Protective Media", *Amer. J. Obstet. Gynecol.* 116:330, 1973.
- Fulka, J., and Motlik, J., "In Vitro Maturation," *Proc. IXth Intl. Congr. Anim. Reprod. Artif. Insem. (Madrid)*, II:55-62, 1980.
- Gardner, R.L., and Edwards, R.G., "Control of Sex Ratio at Full Term in Rabbits by Transferring Sexed Blastocysts", *Nature* 218:346-349, 1968.
- Gatica, R., Boland, M.P., Crosby, T.F., and Gordon, I., "Micromanipulation of Sheep Morulae to Produce Monozygotic twins", *Theriogenology* 21:555-560, 1984.
- Glass, R.H., Spinkle, A.I., and Pederson, R.A., "Mouse Embryo Attachment to Substratum and Interactions of Trophoblast with Cultured Cells", *J. Exper. Zool.* 208:327, 1979.
- Glass, R.H., "Sex Preselection," *Obst. Gynecol.* 49:122-126, 1977.
- Glass, R.H., and Ericsson, R.J., "Intrauterine Insemination of Isolated Motile Spermatozoa," *Fertil. Steril.* 29:535-538, 1978.

Glaub, J.C., Mills, R.N., and Katz, D.F., "Improved Motility Recovery of Human Spermatozoa After Freeze Preservation Via New Approach", *Fertil. Steril.* 27:1283-1291, 1976.

Godke, R.A., Overskei, T.L., and Voelkel, S.A. "The potential for micromanipulation and embryo transfer in breeding goats", *Proc. Dairy Goat Conf. California State Polytechnic University* (November 3), Pomona, CA 10:3-12, 1984a.

Godke, R.A., Voelkel, S.A., Looney, C.R., and Humes, P.E., "Splitting embryos to produce identical twin calves", *Louisiana Agric.* 27:3-4, 1984b.

Goodeaux, S.D., and Kreider, J.L., "Motility and Fertility of Stallion Spermatozoa Isolated in Bovine Serum Albumin," *Theriogenology* 10:405-414, 1978.

Gordon, I., "Problems and Prospects in Cattle Egg Transfer," *Irish Vet. J.* 29:21-30, 1975.

Gordon, M.J., "Control of Sex Ratio in Rabbits by Electrophoresis of Spermatozoa," *Proc., Natl. Acad. Sci.* 43:913, 1957.

Gordon, J.W., Scangos, G.A., Plotkin, D.J., Barbosa, J.A., and Ruddle, F.H., "Genetic Transformation of Mouse Embryos by Microinjection of Purified DNA", *Proc. Natl. Acad. Sci. USA* 77:7380-7384, 1980.

Goss, H., and Cole, H.H., "Further Studies on the Purification of Mare Gonadotropic Hormone", *Endocrinol.* 26:244-249, 1940.

Graham, E.F., Larson, E.V., and Crabo, B.G., "Freezing and Freeze-Drying Bovine Spermatozoa", *Proc. Vth Tech. Conf. Artif. Insem. Reprod.*, pp. 14-20, 1974.

Griffin, J.L., and Randel, R.D., "Successful Transplant of an Ovary from a Mature Hereford Cow to an Immature Crossbred (Non-related) Heifer," *J. Anim. Sci. (Suppl. 1)* 47:103, 1978.

Gubanich, A.A., "Reproduction: The Next 200 Years". *Phi Kappa Phi J.* (winter), pp. 19-24, 1976.

Guillory, B.M., Pool, S.H., Byrd, E.W., and Godke, R.A., "Effect of Various Concentrations of Prostaglandin- F_2 alpha (THAM) on Bovine Spermatozoa In Vitro", *Theriogenology* 11:100, 1979.

Gurdon, J.B., "The Transplantation of Living Cell Nuclei," *Adv. Morphogenesis* 4:1 1964.

Gurdon, J.B., "The Croonian Lecture-1966: Egg Cytoplasm and Gene Control in Development," *Proc. Royal Soc. B (London)* 198:211-247, 1977.

Gwatkin, R.B.L., *Fertilization Mechanisms in Man and Mammals*, (New York: Plenum Press, 1977).

- Hafs, H.D., "A Research and Development Challenge", Proc. XXVth Ann. Conv. NAAB, pp. 124-132, 1971.
- Hafs, H.D. and Boyd, L.J., "Sex Ratios of Calves from Inseminations after Electrophoresis of Sperm," J. Anim. Sci. 38:603-604, 1974.
- Hafez, E.S.E. and Sugie, T., "Reciprocal Transfer of Cattle and Rabbit Embryos," J. Anim. Sci. 22:30-35, 1963.
- Hammer, R.E., Pursel, V.G., Rexroad, Jr., C.E., Wall, R.J., Bolt, D.J., Ebert, K.M., Palmiter, R.D., and Brinster, R.L., "Production of Transgenic Rabbits, Sheep and Pigs by Microinjection," Nature (London) 315:680-683, 1985.
- Hammer, R.E., Pursel, V.G., Rexroad, C.E., Wall, R.J., Boldt, D.J., Palmiter, R.D., Brinster, R.L., "Genetic Engineering of Mammalian Embryos", J. Anim. Sci. 63:269-278, 1987.
- Hammond, J., and Bhattacharya, P., "Control of Ovulation in the Cow", J. Agric. Sci. 34:1-15, 1944.
- Hancock, J.L. and Hovell, G.J.R., "Egg Transfer in the Sow," J. Reprod. Fertil. 4:195-201, 1962.
- Hare, W.C.D., and Betteridge, K.J., "Relationship of Embryo Sexing to Other Methods of Prenatal Sex Determination in Farm Animals: A Review," Theriogenology 9:27-43, 1978.
- Heape, W., "Preliminary note on the transportation and growth of mammalian ova within a uterine foster mother", Proc. Royal Soc. B (London) 48:457-458, 1891.
- Hensleigh, H.C. and Hunter, A.G., "Influence of FSH and HCG Upon the In Vitro Maturation of Bovine Oocytes," Theriogenology 19:133, 1983.
- Hess, E.A., Teague, H.S., Ludwig, T.M., and Martig, R.C., "Swine Can Be Bred with Frozen Semen", Fm. Home Res. 42:100, 1957.
- Heyman, Y., "Factors affecting the survival of whole and half-embryos transferred in cattle", Theriogenology 23:63-75, 1985.
- Heyman, Y., Camous, S., Fevre, J., Meziou, W., and Martal, J., "Maintenance of the Corpus Luteum after Uterine Transfer of Trophoblastic Vesicles to Cyclic Cows and Ewes," J. Reprod. Fertil. 70:533-540, 1984.
- Humes, P.E., and Godke, R.A., "Embryo transplants: The genetic impact", Intl. Limousin J. (June), pp. 30-32, 44, 1975.
- Humes, P.E. and Godke, R.A., "Genetic Impact of Embryo Transfer in Beef Cattle", Proc. Conf. Artif. Insem. Embryo Transf. in Beef Cattle, NAAB (January 12), Denver, CO, pp. 38-44, 1985.

Hunter, G.L., Bishop, G.P., Adams, C.E., and Rowson, L.E.A., "Successful Long-Distance Aerial Transport of Fertilized Sheep Ova," J. Reprod. Fertil. 3:33, 1962.

Illmensee, K., and Hoppe, H.C., "Nuclear Transplantation in Mus musculus: Developmental Potential of Nuclei from Preimplantation Embryos," Cell 23:9-13, 1981.

Illyes, D.R., Warren, W.R., Baham, A., Kreider, J.L., and Godke, R.A., "Freezing Separated Bovine Spermatozoa," J. Anim. Sci. (Suppl. 1) 45:471, 1977.

Illyes, D.R., Warren, W.R., Baham, A., Kreider, J.L., and Godke, R.A., "Effect of Fructose Supplementation on Separated Bovine Spermatozoa," J. Dairy Sci. (Suppl. 1) 61:151-152, 1978.

Ingram, D.L., and Krohn, D.L., "Factors Influencing the Survival of Ovarian Homografts in Rats," J. Endocrinol. 14:110-120, 1956.

Iritani, A., Kasai, M., Niwa, K., and Song, H.B., "Fertilization In Vitro of Cattle Follicular Oocytes with Injected Spermatozoa Capacitated in a Chemically Defined Medium," J. Reprod. Fertil. 70:487-492, 1984.

Iwamatsu, T., and Yanagimachi, R., "Maturation In Vitro of Ovarian Oocytes of Prepubertal and Adult Hamsters," J. Reprod. Fertil. 45:, 1975.

Kinney, G.M., Pennycook, J.W., Schriver, M.D., Templeton, J.W., and Kraemer, D.C., "Surgical Collection and Transfer of Canine Embryos," Biol. Reprod. (Suppl. 1) 20:96A, 1979.

Kiddy, C.A. and Hafs, H.D. (ed.), Sex Ratio at Birth — Prospects for Control. Symposium of American Society of Animal Science, Champaign, IL (Albany, NY: Boyd Printing, 1971), p. 104.

Kiddy, C.A. and Baily, L.F., "Sex Ratios in Rabbits from Semen Subjected to Altered Atmospheric Pressure. J. Anim. Sci. 37:768-769, 1973.

King, T.J., "Nuclear Transplantation in Amphibia" In: Methods in Cell Physiology, D. M. Prescott (ed.) (New York: Academic Press, 1966), Vol. 2, pp. 1-36.

Kraemer, D.C., Moore, G.T., and Kramen, M.A., "Baboon Infant Produced by Embryo Transfer", Science 192:1246-1247, 1976.

Kraemer, D.C., and Vera Cruz, N.C., "Collection, Gross Characteristics and Freezing of Baboon Semen", J. Reprod. Fertil. 20:345-348, 1969.

Koltzoff, N.K., and Schroder, V.N., "Artificial Control of Sex in the Progeny of Mammals," Nature (London) 131:329, 1933.

Kuzan, F.B., and Wright, Jr., R.W., "Observations on the Development of Bovine Morulae on Various Cellular and Noncellular Substrata," J. Anim. Sci. 54:811-816, 1982a.

Kuzan, F.B., and Wright, Jr., R.W., "Blastocyst Expansion, Hatching, and Attachment of Porcine Embryos Cocultured with Bovine Fibroblasts In vitro," Anim. Reprod. Sci. 5:57-63, 1982b.

Kvasnickii, A.V. "Interbreed transplantation of ova", Sovetsk. Zootekh. 1:36. In: Animal Breeding Abstracts 19:224, 1951.

Lambert, R.D., Sirard, M.A., Benard, C., Beland, R., Rious, J.E., Leclerc, P., Menard, D.P., and Bedoya, M., "In Vitro Fertilization of Bovine Oocytes Matured In Vivo and Collected at Laparoscopy", Theriogenology 25:171-133, 1986.

Lambeth, V.A., Looney, C.R., Voelkel, S.A., Hill, K.G., Jackson, D.A., and Godke, R.A., "Micromanipulation of bovine morulae to produce identical twin offspring", Proc. IInd World Congr. Embryo Transf. in Mammals. (September 20-22), Annecy, France, p. 55, 1982.

Lambeth, V.A., Looney, C.R., Voelkel, S.A., Jackson, D.A., Hill, K.G., and Godke, R.A., "Microsurgery on bovine embryos at the morula stage to produce monozygotic twin calves", Theriogenology 20:85-95, 1983.

Langer, G., Lemberg, E., and Sharf, M., "Artificial Insemination: A Study of 156 Successful Cases", Intl. J. Fertil. 14:232-240, 1969.

Lassalle, B., Courtot, A.M., and Testart J., "In Vitro Fertilization of Hamster and Microinjection of Human Sperm", J. IVF Embryo Transf. 3:69, 1986.

Lawson, R.A.S., Rowson, L.E.A., and Adams, C.E., "The Development of Cow Eggs in Rabbit Oviducts and Their Viability after Re-transfer to Heifers," J. Reprod. Fertil. 28:313-315, 1972.

Lehn-Jensen, H. and Willadsen, S.M., "Deep-freezing of cow "half" and "quarter" embryos", Theriogenology 19:49-54, 1983.

Leibfried, L., and First, N.L., "Characterization of Bovine Follicular Oocytes and Their Ability to Mature In Vitro," J. Anim. Sci. 48:76-86, 1979.

Leibo, S.P., "Water Permeability and Its Activation Energy of Fertilized and Unfertilized Mouse Ova", J. Membrane Biol. 53:179-188, 1980.

Leibo, S.P. "A one-step method for direct non-surgical transfer of frozen-thawed bovine embryos", Proc. IInd World Congr. Embryo Transf. in Mammals. (September 20-22), Annecy, France, p. 97, 1982.

Leibo, S.P. "Field Trial of One-Step Frozen Bovine Embryos Transferred Non-Surgically", Theriogenology 19:139, 1983.

Leibo, S.P. "A One-Step Method for Direct Non-Surgical Transfer of Frozen-Thawed Bovine Embryos", Theriogenology 21:767, 1984.

Leibo, S.P., West, A.W., and Perry, B., "A One-Step Method for Direct Nonsurgical Transfer of Frozen-Thawed Bovine Embryos", I. Basic studies. *Cryobiology* 19:673, 1982a.

Leibo, S.P., West, A.W., and Perry, B., "A One-Step Method for Direct Nonsurgical Transfer of Frozen-Thawed Bovine Embryos", II. Basic studies. *Cryobiology* 19:674, 1982b.

Lenz, R.W., Ball, G.D., Lohse, J.K., First, N.L., and Ax, R.L., Chondroitin Sulfate Facilitates and Acrosome Reaction in Bovine Spermatozoa as Evidenced by Light Microscopy, Electron Microscopy and In Vitro Fertilization", *Biol. Reprod.* 28:683-690, 1983.

Leverage, W.E., Valerio, D.A., Schultz, A.P., Kingsbury, E., and Dorey, C., "Comparative Study on the Freeze Preservation of Spermatozoa. Primate, Bovine and Human", *Lab. Anim. Sci.* 22:882-889, 1972.

Looney, C.R., and Godke, R.A. "Non-surgical Embryo Transplants in Cattle", *Louisiana Agric.* 23:10-12, 1980.

Looney, C.R., and Godke, R.A., "Influence of PGF₂ alpha (THAM) and Cloprostenol Upon Bovine Spermatozoa In Vitro", *Theriogenology* 13:101, 1980.

Looney, C.R., Boutte, B.W., Archbald, L.F., and Godke, R.A., "Comparison of Once Daily and Twice Daily FSH Injections in Superovulation of Beef Cattle", *Theriogenology* 15:13-22, 1981.

Looney, C.R., Jackson, D.A., Voelkel, S.A., and Godke, R.A., "The use of fluorescein diacetate (FDA) to assess viability of bovine embryos in vitro", *Proc. II Ind World Congr. Embryo Transf. in Mammals (September 20-22)*, Annecy, France, p. 56, 1982a.

Looney, C.R., Voelkel, S.A., Jackson, D.A., and Godke, R.A. "In vitro culture of bovine embryos with fluorescein diacetate indicator", *J. Anim. Sci. (Suppl. 1)* 55:62, 1982b.

Lopata, A., Johnston, W.I.H., Leeton, J.F., Muchnicki, D., Talbot, J.M.C., and Wood, C., Collection of Human Oocytes at Laparoscopy and Laparotomy", *Fertil. Steril.* 25:1030-1038, 1974.

Lyon, M., Glenister, P., and Whittingham, D.G., Frozen Storage of Laboratory Animal Embryos, G. H. Zeilmaker (ed.) (Stuttgart: G. Fischer Verlag, 1981).

Lush, J.L., "The Possibility of Sex Control by Artificial Insemination with Centrifuged Spermatozoa," *J. Agric. Res.* 30:893, 1925.

Macpherson, J.W., Chatterjee, S., and Friars, G.W., "Frozen Turkey Semen", *Canadian J. Comp. Med.* 33:37-38, 1969.

Macpherson, J.W., and King, G.J., "Plastic Straws for Frozen Semen (A Field Evaluation)", *Canadian J. Comp. med. Vet. Sci.* 30:109-111, 1966.

- Martin, I.C.A., "The Freezing of Dog Spermatozoa to -79°C ", Res. Vet. Sci. 4:304-314, 1963.
- Markert, C.L. "Fertilization of Mammalian Eggs by Sperm Injection", J. Exper. Zool. 228:195-201, 1983.
- Markert, C.L. "Genetic manipulation of mammalian embryos: Current techniques and their potential usefulness in livestock improvement", Proc. Xth World Congr. Anim. Reprod. Artif. Insem (June 10-14), University of Illinois, Urbana 4(2):13-19, 1984.
- Loskutoff, N.M., Coren, B.R., Barrios, D.R., Bessoudo, E., Bowen, M.J., Stone, G., and Kraemer, D.C., "Gene Injection in Bovine Embryos Facilitated by Centrifugation", Theriogenology 25:168, 1986.
- Marston, J.H., Penn, R., and Sivelle, P.C., "Successful Autotransfer of Tubal Eggs in the Rhesus Monkey (Macaca mulatta)", J. Reprod. Fertil. 49:175-176, 1977.
- Martal, J. Lacroix, M.C., Loudes, C., Saunier, M., and Wintenberger-Torres, S., "Trophoblastin, and Antiluteolytic Protein in Early Pregnancy in Sheep," J. Reprod. Fertil. 56:63-73, 1979.
- Maxwell, W.M.C., Mendoza, G., and White, I.G., "Post-thawing Survival of Motile Ram Sperm after Isolation by Layering on Protein Columns," Theriogenology 21:601-607, 1984.
- McFarland, C.W., (Personal Communication) 1986.
- McFarland, C.W., Rorie, R.W., McNeill, D.R., Voelkel, S.A., Hembry, F.G., and Godke, R.A., "Microsurgery on sheep embryos to produce genetically identical twin lambs", Louisiana Agric. 28:3-4, 1985a.
- McFarland, C.W., Rorie, R.W., Voelkel, S.A., and Godke, R.A., "The use of pronase to successfully remove the zona pellucida from bovine embryos", Theriogenology 23:208, 1985b.
- McGaughey, R.W., "In Vitro Oocyte Maturation," In: Methods in Mammalian Reproduction, J.C. Danilets, Jr. (ed.) (New York: Academic Press, 1978).
- McGrath, J., and Solter, D., "Nuclear Transplantation in the Mouse by Microsurgery and Cell Fusion", Science 220:1300-1302, 1983a.
- McGrath, J., and Solter, D., "Nuclear Transplantation in the Mouse Embryos", J. Exp. Zool. 228:355, 1983b.
- McGrath, J., and Solter, D., "Inability of Mouse Blastomere Nuclei Transferred to Enucleated Zygotes to Support Development In Vitro," Science 226:1317, 1984.
- McKinnell, R.G., "Cloning: Nuclear Transplantation in Amphibia. (Minneapolis, University of Minnesota Press), 1978.

Meinecke-Tillmann, S. and Meinecke, B., "Experimental Chimaeras — Removal of Reproductive Barrier Between Sheep and Goat", *Nature* 307:637-638, 1984.

Mertes, P.C. and Bondioli, K.R., "Effect of Splitting Technique on Pregnancy Rate from Half Embryos", *Theriogenology* 23:209, 1985.

Meryman, H.T. "Drying of Living Mammalian Cells", *Ann. NY Acad. Sci.* 85:729, 1960.

Mettler, L. and Semm, K., "Follicular Puncture for Human Ovum Recovery Via Pelviscopy", *Arch. Androl.* 5:87-89, 1980.

Mintz, B. and Illmensee, K., "Normal Genetically Mosaic Mice Produced from Malignant Teratocarcinoma Cells", *Proc. Natl. Acad. Sci.* 72:3585-3589, 1975.

Mohr, L.R. and Trounson, A.O., "The Use of Fluorescein Diacetate to Assess Embryo Viability in the Mouse," *J. Reprod. Fertil.* 58:189-196, 1980.

Mohr, L.R. and Trounson, A.O., "Freezing and Donation of Human Embryos", *J. IVF Embryo Transf.* 1:127, 1984.

Mohr, L.R., and Trounson, A.O., "Cryopreservation of Human Embryos", *Ann. NY Acad. Sci.* 442:536-543, 1985.

Mohr, L.R., Trounson, A.O., and Freeman, L., "Deep Freezing and Transfer of Human Embryos," *J. IVF Embryo Transf.* 2:1-10, 1985.

Moor, R.M., Cahill, L.P., and Stewart, F., "Ovarian Stimulation of Egg Production as a Limiting Factor of Egg Transfer," *Proc. IXth Intl. Congr. Anim. Reprod. Artif. Insem. (Madrid)*, 1:43-58, 1980.

Moore, N.W., and Bilton, R.J., "Frozen Storage of Embryos of Farm Animals: Progress and Implication", In: *The Freezing of Mammalian Embryos. Ciba Foundation Symposium (Amsterdam: Elsevier, 1977)*, pp. 203-211.

Moustafa, L.A. and Hahn, J., "Experimentelle Erzeugung von Identischen Mausezwillingen", *Dtsch. Tierarztl. Wsch.* 85:242-243, 1978a.

Moustafa, L.A., Hahn, J., and Roselius, R., "Versuche zur Geschlechtsbestimmung an Tage 6 und 7 alten Rinderembryonen", *Berl. Munch. Tierarztl. Wsch.* 91:236-238, 1978b.

Mutter, L.R., Graden, A.P., and Olds, D., "Successful Non-Surgical Bovine Transfer", *AI Digest* 12:3, 1964.

Newcomb, R., Christie, W.B., and Rowson, L.E.A., "Birth of Calves after In Vivo Fertilization of Oocytes Removed from Follicles and Matured In Vitro," *Vet. Rec.* 102:461-462, 1978.

Nicholas, J.S., "Development of Transplanted Rat Eggs", Proc. Soc. Exp. Biol. Med. 30:1111-1113, 1933.

Norris, M.L., (Personal Communication), 1981.

Ogawa, S., Takahashi, H., Mizuno, J. Kashiwazaki, N. Yamane, M., and Narishige, E., "Personal Computer Controlled Microsurgery of Fertilized Eggs and Early Embryos", Theriogenology 25:335-345, 1986.

Oguri, N. and Tsutsumi, Y., "Non-surgical Recovery of Equine Eggs, and an Attempt at Non-Surgical Egg Transfer in Horses", J. Reprod. Fertil. 31:187-195, 1972.

Oguri, N. and Tsutsumi, Y. "Non-Surgical Egg Transfer in Mares", J. Reprod. Fertil. 41:313-320, 1974.

Oguri, N., Tsutsumi, Y. and Hachinohe, Y., "Non-Surgical Transfer of Equine Embryos", In: In Vitro Fertilization and Embryo Transfer. E.S.E. Hafez and K. Semm (ed.), (Lancaster: MPT Press, 1982), pp. 287-295.

Ohno, S., Christian, L.C., Wachtel, S.S., and Koo, G.C., "Hormone-like Role of H-Y Antigen in Bovine Freemartin Gonad," Nature (London) 261: 597-599, 1976.

Ohno, S., Nagai, Y., Ciccarese, S. and Iwata, H., "Testis-Organizing H-Y Antigen and the Primary Sex-Determining Mechanism of Mammals", Rec. Progr. Horm. Res. 35:449-470, 1979.

Ozil, J.-P., Heyman Y., and Renard, J.-P., "Production of Monozygotic Twins by Micromanipulation and Cervical Transfer in the Cow", Vet. Rec. 110:126-127, 1982.

Ozil, J.-P. "Production of Identical Twins by Bisection of Blastocysts in the Cow", J. Reprod. Fertil. 69:463-468, 1983.

Palmiter, R.D., Brinster, R.L., Hammer, R.E., Trumbauer, M.E., Rosenfeld, M.G., Birnberg, N.C., and Evans, R.M., "Dramatic Growth of Mice Developed from Eggs Microinjected with Metallothionein-Growth Hormone Fusion Gene", Nature (London) 300:611-615, 1982.

Palmiter, R.D., Norstedt, G., Gelinis, R.E., Hammer, R.E., and Brinster, R.L., "Metallothionein-human GH fusion genes stimulate growth of mice", Science 222:809, 1983.

Parkening, T.A., Fabricant, J.D., Heussner, J.C., Collins, T.J., and Smith, E.R., "Orthotopic Ovarian Transplantations in Young and Aged Mice," Exper. Gerontol. 19:53, 1984.

Parrott, D.M.V., "Orthotopic Ovarian Grafts in the Golden Hamster," J. Endocrinol. 19:126, 1959.

Parrish, J.J., Susko-Parrish, J.L., and First, N.L., "Effect of Heparin and Chondroitin Sulfate on the Acrosome Reaction and Fertility of Bovine Sperm In Vitro", Theriogenology 24:537-550, 1985.

Parrish, J.J., Susko-Parrish, J.L., Leibfried-Rutledge, M.L., Cristser, E.S., Eyestone, W.H., and First, N.L., Bovine In Vitro Fertilization with frozen-thawed semen", Theriogenology 25:591-600, 1986.

Parrott, D.M.V., "The Fertility of Mice with Orthotopic Ovarian Grafts Derived from Frozen Tissue", J. Reprod. Fertil. 1:230-241, 1960.

Peel, C.J., Bauman, D.E., Gorewit, R.G., and Sniffen, C.L., "Effect of Exogenous Growth Hormone on Lactation Performance in High Yielding Dairy Cows", J. Nutr. 111:1662-1671, 1981.

Pendleton, R.J., Youngs, C.R., Rorie, R.W., Pool, S.A., Memon, M.A. and Godke, R.A., "Frozen Embryo Kids Born at LSU," Louisiana Agric. 30:16, 17,23, 1986a.

Pendleton, R.J., Youngs, C.R., Rorie, R.W., Memon, M.A., and Godke, R.A., "Goat Offspring Born from a Split Embryo. Louisiana Agric. 29:6-7, 1986b.

Perry, E.J., (ed.), In: The Artificial Insemination of Farm Animals, 4th ed., (New Brunswick, NJ: Rutgers University Press, 1968), p. 5.

Pighills, E., Hancock, J.L., and Hall, J.G., "Attempted induction of chimaerism in sheep", J. Reprod. Fertil. 17:543-547, 1968.

Plante, C., Bousquet, D., Guay, P., Goff, A.K., and King, W.A., "Luteotrophic Factor Secreted by Bovine Embryonic Tissue," Theriogenology 23:217, 1985.

Polge, C., Smith, A.U., and Parkes, A.S., "Revival of Spermatozoa after Vitrification and Dehydration at Low Temperatures", Nature (London) 164:666-668, 1949.

Polge, C., and Rowson, L.E.A., "Fertilizing Capacity of Bull Spermatozoa After Freezing at -79°C", Nature (London) 169:626-627, 1952.

Pool, S.H., Blakewood, E.G., Rorie, R.W., McFarland, C.W., and Godke, R.A., "The Use of a Household Refrigerator for Short-Term Storage of Bovine Embryos", Theriogenology 25:184, 1986.

Pool, S.H., Rorie, R.W., Pendleton, R.J., Menino, A.R., White, K.L., and Godke, R.A., "Culture of Early-Stage Bovine Embryos Inside Day-13 Pre-culture Trophoblastic Vesicles. Proc. Vth World Congr. In Vitro Fertil. Embryo Transf. (Norfolk, Virginia) (In press) (1987).

Pope, C.E., and Pope, V.Z., (Personal Communication), 1983.

Pope, C.E., Pope, V.Z., and Beck, L.R., "Nonsurgical Recovery of Uterine Embryos in the Baboon", Biol. Reprod. 23:657-662, 1980.

Polzin, V.J., Anderson, D.L., Anderson, G.B., Bon Durrant, R.H., Butler, J.E., Pashen, R.L., Penedo, M.C.T., and Rowe, J.D., "Production of Sheep-Goat Chimeras by Blastocyst Injection", *Theriogenology* 25:183, 1986.

Purdy, J.M., "Fertilization and Preimplantation Growth In Vitro", In: *Human Conception In Vitro*. R.G. Edwards and J.M. Purdy (ed.) (London: Academic Press, 1981), pp. 135-156.

Rall, W.F., and Fahy, G.M., "Ice-Free Cryopreservation of Mouse Embryos at -196°C by Vitrification", *Nature* 313:573-575, 1985.

Rexroad, Jr., C.E., and Powell, A.M., "Co-culture of Sheep Ova and Cells From Sheep Oviduct", *Theriogenology* 25:187, 1986.

Rice, V.A., Andrews, F.N., Warwick, E.J., and Legates, J.E., (ed.), In: *Breeding and Improvement of Farm Animals*. (New York: McGraw Hill, 1957), p. 160.

Rimington, C., and Rowlands, I.W., "Serum Gonadotrophin: II. Further Purification of the Active Material", *Biolchem. J.* 38:54-60, 1944.

Robl, J.M., Prather, R., Barnes, F., Eyestone, W., Northey, Gilligan, B. and First, N.L., "Nuclear Transplantation in Bovine Embryos", *J. Am. Sci.* 64:642-647, 1987.

Rorie, R.W., McFarland, C.W., Overskei, T.L., Voelkel, S.A., and Godke, R.A., "A new method of splitting embryos without the use of a commercial micromanipulator unit", *Theriogenology* 23:224, 1985b.

Rorie, R.W., Voelkel, S.A., McFarland, C.W., Southern, L.L., and Godke, R.A., "Micromanipulation of day-6 porcine embryos to produce split-embryo piglets", *Theriogenology* 23:225, 1985a.

Rorie, R.W., Pendleton, R.J., Pool, S.H., White, K.L., and Godke, R.A., "Cryopreservation of Bovine Trophoblastic Vesicles", *Theriogenology* 27:272, 1987a.

Rorie, R.W., Pendleton, R.J., White, K.L., and Godke, R.A., "An Attempt to Produce Bovine Trophoblastic Vesicles from Embryonic Single Cell Suspensions", *Theriogenology* 27:273, 1987b.

Rorie, R.W., Pendleton, R.J., Pool, S.H., Youngs, C.R., and Godke, R.A., "The Viability of 'Half' Embryos Produced Before and After Liquid Nitrogen Freezing", In: *Future Aspects in Human In Vitro Fertilization*. W. Feichtinger and P. Kemeter (ed.), (Heidelberg: Springer-Verlag, 1987c) pp. 26-35.

Rorie, R.W., Pool, S.H., Pendleton, R., White, K.L., and Godke, R.A., "The Use of Micromanipulation and Cellular Aggregation to Produce Chimeric Bovine Embryos," *Proc., Congr. Peruano de Genetica* (December 8-13) Lima, Peru, p. 45, 1986.

- Ross, A., Robinson, J.A. and Evans, H.J., "Failure to Confirm Separation of X- and Y-bearing Human Sperm Using BSA Gradients," *Nature (London)* 253:354-355, 1975.
- Rossant, J., Croy, B.A., Chapman, V.M., Siracusa, L., and Clark, D.A. "Interspecific Chimeras in Mammals: A New Experimental System", *J. Anim. Sci.* 55:1241-1248, 1982.
- Rottman, O.J. "New Perspectives in Animal Breeding by Embryo Manipulation", *Livestock Prod. Sci.* 10:215-221, 1983.
- Roussel, J.D. and Austin, C.R., "Preservation of Primate Spermatozoa by Freezing", *J. Reprod. Fertil.* 13:333-335, 1967.
- Rowe, R.F., Del Campo, M.R., Eilts, C.L., French, L.R., Winch, W.P., and Ginther, O.J., "A single cannula for non-surgical collection of ova from cattle", *Theriogenology* 6:471-483, 1976.
- Rowlands, I.W., "The Production of Ovulation in the Immature Rat," *J. Endocrinol.* 3:384, 1944.
- Rowlands, I.W., "Artificial Insemination of Mammals in Captivity", *Intl. Zoo Yearbook* 5:105-106, 1964.
- Rowson, L.E.A., "Methods of Inducing Multiple Ovulation in Cattle," *J. Endocrinol.* 7:260-270, 1951.
- Roy, A., Srivastava, R.K., and Pankey, M.D., "Deep Freezing of Buffalo Semen Diluted and Preserved in Glycine-Egg Yolk Medium", *Indian J. Dairy Sci.* 9:61-62, 1956.
- Rumey, R.E., "Fetal Mouse Oviducts in Tissue and Organ Cultures," *Fertil. Steril.* 20:149-161, 1969.
- Rutledge, J.J., and Seidel, Jr., G.E. "Genetic Engineering and Animal Production", *J. Anim. Sci. (Suppl. 2)* 57:265-272, 1983.
- Sadler, R.M., "The Preservation of Mammalian Spermatozoa by Freezing", *Lab. Pract.* 15:413-417, 1966.
- Salamon, S., "Fertility of Ram Semen After Long-Term Storage", *Proc. IX Intl. Congr. Anim. Reprod. Artif. Insem. (Madrid)* 5:420, 1980.
- Salomon, D.S., and Sherman, M.I., "Implantation and Invasiveness of Mouse Blastocysts on Uterine Monolayers," *Exper. Cell Res.* 90:261-268, 1975.
- Seager, S.W.J., and Platz, C.C., "Semen Collection and Freezing in Captive Wild Mammals. *Proc. VIIIth Intl. Congr. Anim. Reprod. Artif. Insem. (Krakow)* 4:1075-1078, 1976.
- Seidel, F., "The development potential of an isolated blastomere in the 2-cell stage mammalian egg", *Naturwissenschaften* 39:355-356, 1952.

Seidel, Jr., G.E., "Management of reproduction of cattle in the 1990's", 1979 Carl B. and Florence E. King Visiting Scholar Lectures. University of Arkansas, Fayetteville. Special Report No. 77, pp. 51-59, 1980a.

Seidel, Jr., G.E., "Potential applications of cloning in animal industry", Proc. IXth Intl. Cong. Anim. Reprod. Artif. Insem. 2:363, 1980b.

Seidel, Jr., G.E., "Superovulation and Embryo Transfer in Cattle", Science 211:351-358, 1981.

Seidel, Jr., G.E., "Applications of Microsurgery to Mammalian Embryos", Theriogenology 17:23-34, 1982.

Seidel, Jr., G.E., "Are identical twins produced from micromanipulation always identical?" Proc. Conf. Artif. Insem. Embryo Transf. in Beef Cattle, NAAB (January 12), Denver, CO, pp. 50-53, 1985.

Seidel, Jr., G.E., and Seidel, S. M., "Bovine Embryo Transfer: Costs and Success Rates", Adv. Anim. Breeder 26:6, 1978.

Servinc, A., "Experiments on Sex Control by Electrophoretic Separation of Spermatozoa in the Rabbit," J. Reprod. Fertil. 16:7, 1968.

Schilling, E., "Sedimentation as an Approach to the Problem of Separating X- and Y-Chromosome-Bearing Spermatozoa," In: Sex Ratio at Birth-Prospects for Control, C. A. Kiddy and H. D. Hafs (ed.) American Society of Animal Science, Champaign, IL, pp. 76-84, 1971.

Schilling, E., "Experiments in Sedimentation and Centrifugation of Bull Spermatozoa and Sex Ratio of Born Calves," J. Reprod. Fertil. 11:469-472, 1966.

Schrivver, M.D., and Kraemer, D.C., "Embryo Transfer in Domestic Feline", Amer. Assoc. Lab. Anim. Sci., Publ. No. 78-4:12, 1978.

Shaikh, A.A., Minhas, B.S., Bowen, M.J., Westhusin, J.E., and Kraemer, D.C., "Pronucleus formation following microinjection of spermatozoa into baboon ova". Proc. Soc. Study Reprod. Abstract No. 294, 1984.

Sherman, J.K., "Improving Methods of Preservation of Human Spermatozoa by Freezing and Freeze-Drying", Fertil. Steril. 14:49-64, 1963.

Sherman, J.K., "Synopsis of the Use of Frozen Human Semen Since 1964: State of the Art of Human Semen Banking", Fertil. Steril. 24:397-412, 1973.

Sherman, J.K., "Freezing and Freeze-Drying of Human Spermatozoa", Fertil. Steril. 5:357-371, 1954.

Sherman, J.K., "Freezing and Freeze-Drying of Bull Spermatozoa", Amer. J. Physiol. 190:281-286, 1957.

- Sherman, J.K., Frozen Human Semen — Past, Present and Future", Fertil. Steril. 15:485-499, 1964.
- Sherman, J.K., and Bunge, R.G., "Observations on Preservation of Human Spermatozoa at Low Temperatures. Proc. Soc. Exper. Biol. Med. 82:686-688, 1953.
- Silber, S.J., "Transplantation of a Human Testis for Anorchia", Fertil. Steril. 30:181-187, 1978.
- Silber, S.J., and Kelly, J. "Successful Autotransplantation of an Intra-Abdominal Testis to the Scrotum by Microvascular Technique", J. Urol. 115:452-456, 1976.
- Silber, S.J., and Rodriguez-Rigou, L.T., "Pregnancy After Testicular Transplant: Importance of Treating the Couple", Fertil. Steril. 33:454-455, 1980..
- Silbert, J.A., "Large Scale Preservation of Human Semen", Cryobiology 9:556, 1972.
- Sims, D.R., Voelkel, S.A., Looney, C.R., Humes, P.E., and Godke, R.A., "Transplant Lambs Born from Frozen Embryos", Louisiana Agric. 25:4-5, 1982.
- Singh, E.L. and Hare, W.C.D., "The feasibility of sexing bovine morula stage embryos prior to embryo transfer", Theriogenology 14:421-427, 1980.
- Sirard, M.A., Lambert, R.D., Menard, D.P., and Bedoya, M., "In Vitro Fertilization in the Cow: Six Calves are Born From Surgical or Non-surgical Uterine Transfer to Heifers", Theriogenology 25:198, 1985.
- Slade, N.P., Takeda, T., Squires, E.L., and Elsdon, R.P., "Development and viability of frozen-thawed equine embryos", Theriogenology 21:263, 1984b.
- Slade, N.P., Williams, T.J., Squires, E.L., and Seidel, Jr., G.E., "Production of identical twin pregnancies by microsurgical bisection of equine embryos", Proc. Xth World Congr. Anim. Reprod. Artif. Insem. (June 10-14), University of Illinois, Urbana 2:241-243, 1984a.
- Smart, Y.C., Roberts, T.K., Clancy, R.L., and Cripps, A.W., "Early Pregnancy Factor: Its Reproduction-Research Review". Fertil. Steril. 35:397-401, 1981.
- Smidt, D., Steinback J., and Scheven, B., "Modified method for the in vivo recovery of fertilized ova in swine", J. Reprod. Fertil. 10:153-156, 1965.
- Soupart, P., "MGA-M Appearance in Ejaculated Human Sperm," Proceedings, Eighth Annual Meeting of the Society for the Study of Reproduction, Fort Collins, CO. Abstr. No. 133, 1975.

Soupart, P., Anderson, M.L., and Repp, J.E., "Initiation of embryonic development by experimental oocyte fusion", *Theriogenology* 9:102, 1978.

Spearow, J.L. "Mechanism of action of genes controlling reproduction", *Proc. 1983 Edinburgh Workshop on Prolific Sheep. The Genetics of Fecundity*, 1984.

Staigmiller, R.B., and Moor, R.M., "Effect of Follicle Cells on the Maturation and Developmental Competence of Ovine Oocytes Matured Outside the Follicle," *Gamete Res.* 9:221-229, 1984.

Step toe, P.C., and Edwards, R.G., "Birth After the Reimplantation of a Human Embryo", *Lancet* 2:366, 1978.

Step toe, P.C., and Edwards, R.G. (Personal Communications), 1982.

Sugie, T., "Successful transfer of a fertilized bovine egg by non-surgical techniques", *J. Reprod. Fertil.* 10:197-201, 1965.

Sullivan, J.J. and Elliott, F.I., "Bull Fertility as Affected by an Interaction Between Motile Spermatozoa Concentration and Fertility Level in Artificial Insemination", *Proc. Vith Intl. Congr. Anim. Reprod. Fertil. Insem. (Paris)* 2:1307-1313, 1968.

Summers, P.M., Shelton, J.N., and Bell, K., "Synthesis of primary *Bos tarus* - *Bos indicus* chimaeric calves", *Anim. Reprod. Sci.* 6:91-102, 1983.

Takeda, T., Elsdén, R.P., and Squires, E.L. "In vitro and in vivo development of frozen-thawed equine embryos", *Proc. Xth Intl. Congr. Anim. Reprod. Artif. Insem. (June 10-14) University of Illinois, Urbana* 2:246-248, 1984a.

Takeda, T., Elsdén, R.P., and Seidel, Jr., G.E., "Cryopreservation of Mouse Embryos by Direct Plunging into Liquid Nitrogen," *Theriogenology* 21:266, 1984b.

Takahashi, Y., and Kanagawa, H., "Quick Freezing of Mouse Embryos by Direct Plunge into Liquid Nitrogen Vapor: Effects of Sugars," *Japanese J. Vet. Res.* 33:141-144, 1985.

Tarkowski, A.K. "Mouse Chimaeras Developed from Fused Eggs", *Nature* 190:857-860, 1961.

Thadani, V.M., "A study of heterospecific sperm-egg interactions in the rat, mouse and deer mouse using in vitro fertilization and sperm injection", *J. Exper. Zool.* 212:435, 1980.

Thompson, D.L., Godke, R.A., and Walker, M.P., "Increasing Testicular Size and Spermatogenic Potential of Bulls by Active Immunization Against Testosterone", *Adv. Anim. Breeder* 3:4,9, 1983.

- Thibault, C.G., "Final Stages of Mammalian Oocyte Maturation," In: Oogenesis, J. D. Biggers and A. W. Schuetz (ed.) (Baltimore: University Park Press, 1972), pp. 397-411.
- Toyoda, Y., and Chang, M.C., "Fertilization of Rat Eggs In Vitro by Epididymal Spermatozoa and the Development of Eggs Following Transfer," J. Reprod. Fertil. 36:9-22, 1974.
- Trounson, A., and Mohr, L., "Human Pregnancy Following Cryopreservation, Thawing and Transfer of an Eight-Cell Embryo", Nature 305:707-709, 1983.
- Trounson, A.O., and Moore, N.W., "The Survival and Development of Sheep Eggs Following Complete or Partial Removal of the Zona Pellucida", J. Reprod. Fertil. 41:97-105, 1974.
- Tsunoda, Y., Soma, T., and Sugie, T., "Inhibition of Fertilization by Zona Pellucida Antibody", In: In Vitro Fertilization and Embryo Transfer. E.S.E. Hafez and K. Semm (ed.), (Lancaster: MTP Press, 1982), pp. 117-127.
- Tsunoda, Y., Wakasu, M., Yasui, T., and Sugie, T. "Micromanipulation and freezing of goat embryos", Proc. Xth Intl. Congr. Anim. Reprod. Artif. Insem. (June 10-14), University of Illinois, Urbana 2:251, 1984.
- Tucker, E.M., Moor, R.M., and Rowson, L.E.A., "Tetraparental sheep chimeras induced by blastomere transplantation. Changes in blood type with age", Immunology 26:613-621, 1974.
- Tyler, E.T., "The Clinical Use of Frozen Semen Banks", Fertil. Steril. 24:413, 1973.
- Tyndale-Biscoe, C.H., "Blastocyst Transfer in the Marsupial, Setonix brachyurus", J. Reprod. Fertil 6:41-48, 1963.
- Tyndale-Biscoe, C.H., "Resumption of development by quiescent blastocysts transferred to primed, ovariectomized recipients in the marsupial, Macropus euginii", J. Reprod. Fertil. 23:25-32, 1970.
- Uehara, T., and Yanagimachi, R., "Microsurgical injection of spermatozoa into hamster eggs with subsequent transformation of sperm nuclei into male pronuclei", Biol. Reprod. 15:467-470, 1976.
- Viker, S.D., Voelkel, S.A., Rorie, R., Lambeth, V.A., and Godke, R.A., "A new method for freezing embryos using microprocessor-controlled thermoelectric cells", Proc. IIIrd World Conf. In Vitro Fertil. Embryo Transf. (May 14-17), Helsinki, Finland, p. 128, 1984a.
- Viker, S.D., Voelkel, S.A., and Godke, R.A., (Unpublished Data), 1984b.
- Viker, S.D., Voelkel, S.A., Hill, K.G., Jochle, W., and Godke, R.A., "Solcoseryl treatment of day-7 bovine embryos in vitro". Theriogenology 21:270, 1984c.

- Vincent, C.K., Robison, O.W., and Ulberg, L.C., "A technique for reciprocal embryo transfer in swine", *J. Anim. Sci.* 23:1084-1088, 1964.
- Voelkel, S.A., Amborski, G.F., Hill, K.G., and Godke, R.A., "Use of a Uterine-Cell Monolayer Culture System for Micromanipulated Bovine Embryos", *Theriogenology* 24:271-281, 1985.
- Voelkel, S.A., Humes, P.E. and Godke, R.A., "Pregnancy rates resulting from non-surgical transfer of micromanipulated bovine embryos". *Proc. Xth World Congr. Anim. Reprod. Artif. Insem.* (June 10-14), University of Illinois, Urbana 2:251-253, 1984c.
- Voelkel, S.A., Stuckey, K.W., Looney, C.R., Enright, F.M., Humes, P.E., and Godke, R.A., "An attempt to isolate *Brucella abortus* from uterine flushings of brucellosis-reactor donor cattle", *Theriogenology* 19:355-366, 1983.
- Voelkel, S.A., Viker, S.D., Humes, P.E., and Godke, R.A., "Micromanipulation and non-surgical transfer of bovine embryos", *J. Anim. Sci.* (Suppl. 1) 59:393, 1984d.
- Voelkel, S.A., Viker, S.D., Johnson, C.A., Hill, K.G., Humes P.E., and Godke, R.A., "First calves produced from quartered embryo", *Louisiana Agric.* 27:5, 1984e.
- Voelkel, S.A., Viker, S.D., Johnson, C.A., Hill, K.G., Humes P.E., and Godke, R.A. Multiple embryo transplant offspring produced from quartering a bovine embryo at the morulae stage. *Vet Rec.* 117:528-530, 1985b.
- Voelkel, S.A., Viker, S.D., Lambeth, V.A., and Godke, R.A. "A new approach to freezing bovine embryos without the use of liquid cooling agents", *Theriogenology* 21:272, 1984a.
- Vogelsang, S.G., Sorensen, Jr., A.M., and Kraemer, D.C., "Fertility of donor mares following non-surgical collection of embryos", *J. Anim. Sci.* (Suppl. 1) 47:397, 1978.
- Wachtel, S.S., "H-Y Antigen in the Study of Sex Determination and Control of Sex Ratio", *Theriogenology* 21:18-28, 1984.
- Wachtel, S.S., Koo, G.C., Zuckerman, E., Hammerling, U., Scheid, M., and Boyse, E.A., "Serological Crossreactivity between H-Y (Male) Antigens of Mouse and Man," *Proc. Natl. Acad. Sci., USA*, 71:1215-1218, 1974.
- Wagner, T.E., Hoppe, P.C., Jollick, J.D., Scholl, D.R., Hodinka, R.L., and Gault, J.B., "Microinjection of a rabbit beta-globin gene into zygotes and its subsequent expression in adult mice and their offspring", *Proc. Natl. Acad. Sci. USA* 78:6376-6380, 1981.
- Wagner, T.E., Murray, F.A., Minhas B., and Kraemer, D.C., "The Possibility of Transgenic Livestock", *Theriogenology* 21:29-44, 1984.

- Walker, M.P., Thompson, Jr., D.L., Godke, R.A., and Honey, P.G., "Active Immunization of Prepubertal Bulls Against Testosterone: Seminal and Testicular Characteristics After Puberty", *Theriogenology* 22:269-278, 1984.
- Warwick, B.L. and Berry, R.O., "Inter-Generic and Intra-Specific Embryo Transfers", *J. Heredity* 40:297-303, 1949.
- Warwick, B.L., Berry, R.O., and Horlacher, W.R., "Results of mating rams to Angora female goats", *Proc. 27th Ann. Meetings, Amer. Soc. Anim. Prod.*, pp. 225-227, 1934.
- Westhusin, M.E., Anderson, J.G., Harms, P.G., and Kraemer, D.C., "Micro-injection of Spermatozoa Into Bovine Eggs", *Theriogenology* 21:274, 1984.
- White, K.L., Anderson, G.B., and BonDurant, R.H., "Expression of a Male-Specific Histocompatibility Protein on Various Stages of Preimplantation Bovine Embryos," *Biol. Reprod.* (Submitted), 1987d.
- White, K.L., Anderson, G.B., BonDurant, R.H., Donahue, S., and Pashen, R.L., "Viability of Bisected Bovine Embryos after Detection of H-Y Antigen," *Theriogenology* 27:(In press), 1987a.
- White, K.L., Anderson, G.B., Berger, P.J., BonDurant, R.H., and Pashen, R.L., "Expression of Male-Specific Factor (H-Y Antigen) on Preimplantation Porcine Embryos," *Proc. Amer. Soc. Anim. Sci.* (August), 1985.
- White, K.L., Anderson, G.B., Berger, P.J., BonDurant, R.H., and Pashen, R.L., "Identification of a Male-Specific Histocompatibility Protein on Preimplantation Porcine Embryos," *J. Reprod. Fertil.* (Submitted), 1987c.
- White, K.L., Anderson, G.B., Pashen, R.L. and BonDurant, R.H., "Detection of Histocompatibility-Y (H-Y) Antigen: Identification of the Sex of Preimplantation Ovine Embryos," *J. Reprod. Immunol.* (In press), 1987b.
- White, K.L., Bradbury, M.W., Anderson, G.B., and BonDurant, R.H., "Immunofluorescent Detection of a Male Specific Factor on Preimplantation Bovine Embryos," *Theriogenology* 21:275, 1984.
- White, K.L., Lindner, G.M., Anderson, G.B., and BonDurant, R.H., "Survival after Transfer of "Sexed" Mouse Embryos Exposed to H-Y Antisera", *Theriogenology* 18:655, 1982.
- White, K.L., Lindner, G.M., Anderson, G.B., and BonDurant, R.H., "Cytolytic and Fluorescent Detection of H-Y Antigen on Preimplantation Mouse Embryos", *Theriogenology* 19:701-705, 1983.
- White, L.M., Beal, W.E., and Saacke, R.G., "Isolation of Highly Motile, Morphologically Normal Bovine Spermatozoa. Virginia Polytechnic University Livestock Res. Rep., (Animal Science No. 2), pp. 237-240, 1982.
- Whittingham, D.G., "Fertilization of Mouse Eggs In Vitro," *Nature* (London) 220:592-593, 1968.

- Whittingham, D.G., "The Viability of Frozen-Thawed Mouse Blastocysts", *J. Reprod. Fertil.* 37:159-162, 1974.
- Whittingham, D.G., "Survival of Rat Embryos After Freezing and Thawing", *J. Reprod. Fertil.* 43:575-578, 1975a.
- Whittingham, D.G., "Fertilization, Early Development and Storage of Mammalian Ova In Vitro", In: *Symposium on Early Mammalian Development*, British Society of Developmental Biology, M. Balls and A. E. Wild, (London: Cambridge University Press, 1975b), pp. 1-24.
- Whittingham, D.G., Leibo, S.P., and Mazur, P., "Survival of Mouse Embryos Frozen to -196°C and -269°C ", *Science* 178:411-414, 1972.
- Wiemer, K.E., Amborski, G.F., Denniston, R.S., White, K.L., and Godke, R.A., "Use of a Hormone-Treated Fetal Uterine Fibroblast Monolayer System for In Vitro Culture of Bovine Embryos," *Theriogenology* 27:294, 1987.
- Willadsen, S.M. "A method for culture of micromanipulated sheep embryos and its use to produce monozygotic twins", *Nature (London)* 277:298-300, 1979.
- Willadsen, S.M. "The viability of early cleavage stages containing half the normal number of blastomeres in the sheep", *J. Reprod. Fertil.* 59:357-362, 1980.
- Willadsen, S.M. "The development capacity of blastomeres from 4- and 8-cell sheep embryos", *J. Embryol. Exper. Morph.* 65:165-172, 1981.
- Willadsen, S.M. "Micromanipulation of embryos of the large domestic species", In: *Mammalian Egg Transfer*. C.E. Adams (ed.), Boca Raton, FL: CRC Press, 1982), pp. 185-210.
- Willadsen, S.M., "Nuclear Transplantation in Sheep Embryos," *Nature (London)* 320:63-65, 1986.
- Willadsen, S.M. and Fehilly, C.B., "The Development Potential and Regulatory Capacity of Blastomeres from Two-, Four- and Eight-Cell Sheep Embryos", In: *Fertilization of the Human Egg In Vitro*. H.M. Bier and H.R. Lindner (ed.) (Heidelberg:Springer-Verlag, 1983), pp. 353-357.
- Willadsen, S.M. and Godke, R.A., "A Simple Procedure for the Production of Identical Sheep Twins", *Vet. Rec.* 114:240-243, 1984.
- Willadsen, S.M., Lehn-Jensen, H., Fehilly, C.B., and Newcomb, R., "The production of monozygotic twins of preselected parentage by micromanipulation of non-surgically collected cow embryos", *Theriogenology* 15:23-29, 1981.
- Willadsen, S.M., Pashen, R.L., and Allen, W.R., "Micromanipulation of Horse Embryos", *Soc. Study Fertil., Ann. Conf.* p. 28, 1980.

- Willadsen, S.M., and Polge, C., "Attempts to Produce Monozygotic Quadruplets in Cattle by Blastomere Separation", *Vet. Rec.* 108:211-213, 1981.
- Willadsen, S.M., Polge, C., Rowson, L.E.A., and Moor, R.M., "Preservation of Sheep Embryos in Liquid Nitrogen", *Cryobiology* 11:560, 1974.
- Willadsen, S.M., Polge, C., Rowson, L.E.A., and Moor, R.M., "Deep Freezing of Sheep Embryos", *J. Reprod. Fertil.* 46:151-154, 1976.
- Willadsen, S.M., Polge, C., Trounson A.O., and Rowson, L.E.A., "Transplantation of sheep and cattle embryos after storage at -196°C ", In: *The Freezing of Mammalian Embryos*, Ciba Foundation Symposium, No. 52. K. Elliott and J. Whelan (ed.) (North Holland, Amsterdam: Elsevier/Excerpta Medica, 1977), pp. 190-194.
- Willet, E.L., Black, W.G., Casida, L.E., Stone, W.H., Buckner, P.J., "Successful Transplant of a Fertilized Bovine Ovum", *Science NY* 113:247. 1951.
- Williams, T.J., Elsdon, R.P., and Seidel, Jr., G.E., "Identical twin bovine pregnancies derived from bisected embryos", *Theriogenology* 17: 114, 1982.
- Williams, T.J., Elsdon, R.P., and Seidel, Jr., G.E., "Pregnancy rates with bisected bovine embryos", *Theriogenology* 22:521-531, 1984.
- Williams, T.J., and Johnson, S.E., "Quick-Freeze of Day Four Mouse Embryos", *Theriogenology* 23:235, 1985.
- Wilmut, I. and Rowson, L.E.A., "Experiments on the low temperature preservation of cow embryos", *Vet. Rec.* 92:686-690, 1973.
- Winterberger-Torres, S. and Popescu, P.C., "Transfer of Cow Blastocysts After Sexing", *Theriogenology* 14:309-328, 1980.
- Wolf, N.B., "New Hope for Exotic Species," *Bio. Sci.* 36:594-597, 1986.
- Wright, R.W., and Bondioli, K.R., "Aspects of *In Vitro* Fertilization and Embryo Culture in Domestic Animals," *J. Anim. Sci.* 53:702-729, 1981.
- Yang, X., Battista, M., and Foote, R.H., "A Study of Methods for Bisecting Rabbit Embryos", *Theriogenology* 23:238, 1985.
- Youngs, C.R., Pendleton, R.J., Rorie, R.W., and Godke, R.A., "The Use of a Computerized Image Analysis System for Photometric and Morphometric Measurements of Bovine Preimplantation Embryos", *Theriogenology* 27:299, 1987.
- Zavos, P.M., "Preconception Sex Determination via Intravaginal Administration of H-Y Antisera in Rabbits", *Theriogenology* 20:235-240, 1983.

Zorn, J.R., Dubois, P., Janssens, Y., Tanguy, G., Guichard, A., and Cedard, L., "Estradiol and Progesterone in Siliva, a Non Invasive Method of Monitoring Ovarian Hyperstimulation for IVF", J. IVF Embryo Transf. 1:151, 1984.

Table 1
Group methods on fertilization and embryonic growth in vitro

	Bourn Hall	Gothenberg	Kiel	Melbourne — Monash	Melbourne — Royal Women's Hospital
Medium Type	Earle's	Tyrode's	F10	F10, Earle's, T6, Hoppe/Pitts	F10 + Ca lactate
Conditions in medium	pH 7.3, 285 mOsmol Kg ⁻¹	pH 7.4, 285 mOsmol Kg ⁻¹			pH 7.35, 280 mOsmol Kg ⁻¹
Macromolecule	Patient's serum (8%)	Patient's serum (15%)	Serum (15%)	Serum (7.5%)	Serum (10%)
Spermatozoa					
Time obtained	Before laparoscopy	Before laparoscopy	3 hr before insemination	2 hr before insemination	2 hr before insemination
Wash	Culture medium	Culture medium			2 washes in culture medium
Treatment of oocyte before insemination	Incubated 1-12 hr	Incubated 2-6 hr	Incubated 5-6 hr	Incubated 5-6 hr	Incubated 4-6 hr
Conditions of culture					
Incubation	Paraffin/tube	Paraffin/tube	Tube	Tube	Tube
Gas phase	5:5:90	5:5:90	5:5:90	5:5:90, 5:10:95 or 5:95	5:5:90
No. sperm/ml	1-2 × 10 ³ living	5 × 10 ³ total	2-5 × 10 ³		2-5 × 10 ³
Time of transfer to growth medium					
Medium					
Type	12-24 hr	20 hr	15-20 hr	16 hr	18 hr
Conditions in medium	Earle's	F10	F10	As above	F10 + Ca lactate
Macromolecule	As above Patient's serum (15%)	Serum (15%)	As above Serum (15%)	Serum (15%)	As above Serum (15%)
Conditions of culture					
Incubation	Tube usually	Tube	Tube	Tube	Tube
Gas phase	5:5:90	5:5:90	5:5:90	As above	5:5:90
Observed development	Cleavage usually	Cleavage	Cleavage	Cleavage usually	Pronuclei and cleavage
	4 cell-blastocyst	Stage when embryo replaced			4-8 cell
		2-8 cell		2-8 cell	

Table 1 (continued)

	Norfolk	Oldham	Kershaws	Paris—Clamart	Paris—Sèvres	Vienna
Medium			<i>Fertilization</i>			
Type	F10		Tyrodé's, Earle's	B2	B2	F10 + lactate + K ⁺
Conditions in medium			pH 7.3, 285 mOsmol Kg ⁻¹	pH 7.5, 265-280 mOsmol Kg ⁻¹	pH 7.4, 280 mOsmol Kg ⁻¹	pH 7.4-7.5, 276 mOsmol Kg ⁻¹
Macromolecule	Albumin		Patient's serum (8%)	Albumin	Human serum albumin	Albumin
Spermatozoa						
Time obtained	2 hr before insemination		Before laparoscopy	Before laparoscopy	Before laparoscopy	Before laparoscopy
Wash			Culture medium Incubated 0-15 hr	Culture medium Not pre-incubated	Culture medium Incubated 1-2 hr	Culture medium Incubated 2-4 hr
Treatment of oocyte before insemination	Incubated 5-6 hr					
Conditions of culture						
Incubation			Paraffin/tube	Tube	Tube	Tube
Gas phase			5:5:90	5:5:90	5:5:90	5:5:90
No. sperm/ml	5:95		10 ⁶ total	2-10 × 10 ³ living	5 × 10 ³	10 ⁶
			<i>Cleavage</i>			
Time of transfer to growth medium	6-8 hr		12-24 hr	6-23 hr	12-24 hr	12-16 hr
Medium						
Type	F10		Earle's, F10	B2	B2	As above
Conditions in medium			As above	As above	As above	As above
Macromolecule			Patient's serum (15%)	Albumin or serum (15%)	Patient's serum (15%)	Patient's serum (15%)
Conditions of culture						
Incubation			Tube/paraffin	Tube	Tube	Tube
Gas phase			5:5:90	5:5:90	5:5:90	5:5:90
Observed development	Cleavage		Pronuclei and cleavage	Cleavage	Cleavage	Pronuclei and cleavage
			<i>Stage when embryo replaced</i>			
	4 cell		8 cell-blastocyst	4-10 cell	6-12 cell	2-16 cell

Edwards and Purdy (1981)

Table 2.
Reproduction (The Future)

In an article "Reproduction: The Next 200 Years" written by Alan Gubanich (1976) of the University of Nevada it was predicted the following events will be accomplished in the decades to come:

- Fertilization of Eggs Outside the Body
 - Transplanting Embryos
 - Predetermining the Sex of Offspring
 - Storage of Sperm or Eggs for Future Conception
 - Artificial Body Parts
 - Freezing Live Embryos and Ova for Future Conception
 - Synthesis of Genes to Replace Defective Genes, to Cure Genetic Diseases
 - Synthesis of Genes to Predesign Offspring
 - Regeneration of Body Parts
 - Cloning of Animals or Humans
 - Artificial Creation of Life, and Possibly Test-Tube Babies
-

Adapted from Gubanich (1976)

FRONTIERS OF REPRODUCTIVE RESEARCH

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INTRODUCTION

The field of reproductive medicine is rapidly expanding. New knowledge in ovarian, tubal and neuroendocrine physiologic function is increasing at an astounding rate. This new understanding of the physiologic functioning of the human reproductive process has occurred in part because of good basic science research in large and small animal models but primarily because of the advent of in vitro fertilization and embryo transfer. This technique has allowed scientists and clinicians to view human reproduction in progress. It has become a "physiologic window" through which we can learn and begin to understand the basic aspects of human reproductive function.

The future approach to human infertility is difficult to predict. However, current investigation will certainly lead to other areas of detailed studies in the human. It is our hope that this report will review current studies and demonstrate how these will lead to certain areas of investigation in the next five to ten years.

1.0 The Menstrual Cycle

The "natural" or unstimulated ovarian cycle typically results in the development of a single preovulatory follicle containing a single mature oocyte. This oocyte, arrested in the first meiotic prophase since fetal life, will have resumed meiosis and extruded the first polar body prior to its ovulation at midcycle. The actual controlling mechanisms of this complex process are still not well understood. As research in this area progresses, the phenomenon becomes somewhat clearer. However, the factors that determine which follicle will start development but later undergo atresia and which follicle will mature and ovulate, still remain a mystery.

The basic driving force behind the regulation of the menstrual cycle is hypothalamic GnRH production. GnRH, a decapeptide, is secreted in a pulsatile fashion with the frequency and amplitude of these pulses varying according to the phase of the menstrual cycle. The secretory activity of GnRH is thought to reside primarily in the arcuate nucleus of the hypothalamus. The modulation of this pulse generator is affected by catecholamines and neuropeptides, which, in turn, can be affected by higher CNS function and ovarian steroid production. Because of its short half-life and extremely low levels, GnRH pulsatility has generally been studied by measuring peripheral luteinizing hormone (LH) levels. LH is secreted in pulses which can be detected in the circulation and are thought to correspond to GnRH as evidenced by an increase in LH secretion after administration of Naloxone, an opioid antagonist (Ropert, et al, 1981). Closely associated with opioid inhibition is the dopaminergic system which likewise

appears to inhibit GnRH secretion (Ropert, et al, 1984). LH pulses in the early follicular phase are of low amplitude and frequency with both of these increasing toward the midcycle when both are at a maximum (Filicori, et al, 1986). Later on in luteal phase, LH pulses are of low frequency and high amplitude.

The pituitary responds to the GnRH stimulus by secreting follicle stimulating hormone (FSH) and luteinizing hormone (LH). This response is modulated by GnRH pulse frequency and by feedback mechanisms of ovarian steroids and perhaps follicular regulatory proteins. Both FSH and LH are secreted in pulses which correspond to GnRH stimulation. These are more apparent with LH which has a much shorter half-life in circulation (15 minutes) and thus is usually measured to reflect GnRH pulsatility.

In the early follicular phase, FSH is secreted in response to increasing GnRH frequency and decreasing serum estradiol levels from the previous luteal phase. The FSH induces cyclic AMP production in early antral follicles in the ovary. In turn, this system induces protein synthesis, cell proliferation and follicular growth. By this mechanism, several follicles (a "cohort") are recruited and brought into the picture for possible ovulation (DiZerega & Hodgen, 1981). As the ovary responds to the gonadotropin stimulation by increasing the output of estradiol (E_2), FSH secretion decreases. Follicular fluid inhibin may also play a role in this FSH suppression. This negative feedback is important in the ultimate selection process of the dominant follicle. Interference with this negative feedback can cause several follicles, rather than just one, to grow and reach

ovulatory potential. Clomiphene citrate, an anti-estrogen, acts in this manner by blocking the E_2 receptor at the level of the pituitary and probably the hypothalamus. Thus, more gonadotropins are secreted and multiple follicle production can be accomplished (Adashi, 1984).

It is important to realize that the pituitary can respond positively to GnRH only if the GnRH stimulation is pulsatile, and even then, only if the pulse frequency is in the physiologic range. Excessively high frequency pulsatile or tonic (non-pulsatile) administration of GnRH leads to down-regulation of GnRH receptors and a complete shut down of gonadotropin secretion (Knobil, 1980). This dual response of the pituitary is useful in two ways for IVF. Firstly, GnRH can be administered for ovulation induction but must be given by a pump mechanism. If high doses are used for multiple follicle production, the intravenous route should be utilized as subcutaneous administration can lead to prolonged absorption times and thus, pituitary down-regulation. Secondly, GnRH analogs (agonists or antagonists) can be utilized in a tonic fashion to effect a "medical hypophysectomy" and stop ovarian activity prior to beginning ovarian stimulation for multiple follicular development (Kenigsberg, et al, 1984). This is potentially useful in the resistant polycystic ovary syndrome (PCO) patient with high androgen levels.

The follicular phase ends with the initiation of midcycle events and ovulation. At the level of the pituitary, this consists of decreasing FSH secretion followed by a large release of gonadotropins. Rising E_2 levels in the follicular phase increase

pituitary sensitivity to GnRH as well as induce increased gonadotropin synthesis. The actual signal which triggers the initiation of the surge is unknown, but it is thought to be due to a critical level of E_2 over a sufficient length of time. This theory is supported by the observation that an exogenous estrogen bolus can induce an LH surge in the absence of any other hormones or stimulation. In the endogenous cycle, progesterone (P) increases in the circulation just prior to the midcycle surge and appears to be important in causing the FSH surge and in augmenting the LH output (March, et al, 1979). As the gonadotropin surge begins, both LH and FSH increase dramatically with LH pulse frequency as well as amplitude being augmented. The LH surge then causes several important changes to take place in the ovary. The granulosa cells luteinize and shift their steroidogenesis from E_2 to that of P production. The oocyte resumes meiosis and extrudes the first polar body. This latter action, triggered by LH, is probably mediated by Oocyte Maturation Inhibition (OMI), which has been shown to decrease in the follicular fluid of the developing dominant follicle prior to ovulation but remains in high concentrations in follicles containing immature oocytes (Tsafiri, et al, 1982). Finally, ovulation takes place with an extrusion of the oocyte from the follicle.

In the stimulated cycle, Human Chorionic Gonadotropin (HCG) is frequently used to trigger ovulation and to act as a surrogate LH surge. HCG is a glycoprotein hormone which is very similar to LH and has a high affinity with LH receptors. HCG is capable of inducing all of the changes brought about, in the natural cycle, by LH. We have recently shown that LH surges, in fact, occur

after HCG administration in many cases and augment the total LH bioactivity of the surrogate LH surge (Paulson, et al., 1987).

Premature follicular phase LH release during controlled stimulation for IVF occurs in a small percentage of cases and appears to be caused by superphysiologic levels in E_2 . These aborted or blunted LH surges do not cause ovulation but can disrupt the cycle by affecting oocyte quality resulting in decreased fertilization and/or embryo viability (Vargyas & Marrs, 1986). The use of GnRH analogs may decrease the frequency of these events.

In the natural cycle, the ovary responds to pituitary gonadotropin stimulation by the formation of a dominant follicle which responds to the midcycle gonadotropin surge by releasing a mature oocyte. The single ovulating follicle is the result of the recruitment of a cohort of pre-ovulatory follicles and the subsequent selection of a dominant follicle.

Recruitment is accomplished primarily by FSH. As the corpus luteum regresses at the end of the previous luteal phase, E_2 and P negative feedback diminishes. The pituitary responds by increasing FSH output. Early antral follicles in the ovary respond to the FSH by proliferation of granulosa cells and induction of aromatase activity with a resultant increase in E_2 secretion. Stimulation strategies for IVF which utilize FSH early in the menstrual cycle do so in the hope of recruiting as many pre-antral follicles into the growing cohort as possible.

Selection of the dominant follicle from the cohort is accomplished in the natural cycle by the negative feedback of E_2 upon the pituitary. Developing follicles require continued FSH stimulation in order to escape atresia and regression. The follicle which is the first to respond to gonadotropin stimulation by inducing its own FSH receptors guarantees its own continued response in face of decreasing FSH levels. As the co-dominance of several follicles is the desired endpoint of IVF stimulation, it is of benefit to block this selection process. This can be accomplished with clomiphene citrate blockade of E_2 negative feedback as well as by exogenous gonadotropin administration. By maintaining elevated FSH levels further in the follicular phase, several follicles can be kept from undergoing atresia and attaining ovulatory potential.

In addition to the steroid-gonadotropin feedback loop, the follicular fluid protein hormone inhibin has been suggested to play a role in the regulation of follicular development. Inhibin is a protein which can be isolated from the follicular fluid of several species, including humans (Marrrs, et al, 1984). In the natural cycle, inhibin may play a role in the selection of the dominant follicle by inhibiting FSH release from the pituitary, thus working in cooperation with E_2 feedback. In the stimulated cycle, inhibin may play a role in blocking estrogen-positive feedback, thus preventing the midcycle surge in spite of very elevated E_2 levels (Hodgen, 1982).

There are a number of other regulatory proteins which can be isolated from follicular fluid and their role in the natural and stimulated cycle is currently under investigation (DiZerega, 1983;

DiZerega, 1983). These regulatory proteins probably act by intrafollicular or paracrine mechanisms in the selection and maturation process of the follicle. Their precise role in the stimulated and natural cycles remains to be determined.

2.0 Production, Induction, Evaluation and Delivery of Gonadotropins Which Control Ovarian Function in the Woman

Clinical Applications for luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in controlling and modulating ovarian function in a broad range of species have long been recognized, and our understanding of mechanisms of the action of these pituitary gonadotropins has increased exponentially since the functional interrelationships between the hypothalamus and anterior pituitary were first elucidated in the 1940's. Results of these studies have provided a basic understanding of the origin and effects of LH and FSH in mammalian reproduction, and have been reviewed in detail (Chappel, Ulloa-Aguirre & Contifarís, 1983). However, despite the apparent plethora of information on actions of gonadotropins in ovarian physiology, the complex endocrine interrelationships within the pituitary-ovarian axis and the cause(s) of variable responses of subfertile women to exogenous gonadotropins remain poorly understood. Furthermore, the more significant problems associated with controlled multiple follicle recruitment in subfertile women (e.g. spontaneous LH surge, luteal phase deficiency, development of single dominant follicle, induction of asynchronous crops of follicles) remain largely unresolved. Therefore, aspects of current and proposed investigations on the role of gonadotropins in induction of multiple follicular development in subfertile women, and the

projected impact of this work in infertility management, are outlined below. It is speculated that developments in the areas discussed shall resolve the major shortcomings related to use of gonadotropins in reproductive medicine and have a major impact on the efficacy of treatment of the infertile couple.

2.1 Production of gonadotropins used in infertility management

Most of the exogenous gonadotropin used for ovarian stimulation in women in the USA is derived from urine collected from post-menopausal women (human menopausal gonadotropin, Pergonal, Serono Laboratories, Inc., Randolph, MA; human urinary follitropin, Metrodin, Serono). Current techniques to isolate and purify LH and FSH from urine involve protein precipitation (usually by ethanol, acetone or ammonium sulfate), separation of proteins by charge, and final purification by molecular weight fractionation (Reichert et al., 1973; Reichert & Ward, 1974; Beyer et al., 1979). During the past decade, applications of urinary gonadotropin extracts in the management of human infertility, including ovarian hyperstimulation in in vitro fertilization patients, have increased rapidly. Demand, therefore, appears to have accelerated at a faster rate than production by the pharmaceutical companies, with a resultant diminution and uncertainty of supply and quality of these preparations, particularly human menopausal gonadotropins. In addition, experiences of a large number of IVF centers using proprietary hMG preparations have revealed evidential variation in the biopotency of batches of hMG from the same manufacturer, and between manufacturers. These differences are consistent with evidence that all immunoactive gonadotropin in urine of post-menopausal

women (and others) is not bioactive, and that gonadotropins secreted by the pituitary are released as families of proteins, some fractions being more bioactive than others (Robertson et al., 1977; Zaidi et al., 1981). The relative abundance of bioactive gonadotropins, relative to total gonadotropin released by the human pituitary, also appears to decline with advancing age and this is in accord with low ratios of bioactive to immunoactive gonadotropin in urine of post-menopausal women. Variations in the bioactivity of urinary gonadotropins are reflected by unpredictable responses of patients to "fixed doses" of hMG (for example), and this widespread experience is of major clinical interest.

With respect to the above concerns, encouraging progress toward in vitro synthesis of homogenous gonadotropin preparations of high biological potency is being made. The structures of LH and FSH were first elucidated in 1981 (Pierce & Parsons, 1981), leading to more recent investigation of regulation of mRNA molecules coding for the respective glycoprotein subunits in the pituitary gonadotropes (Alexander & Miller, 1982). It has now been established that biosyntheses of the subunits of LH and FSH are regulated by different mRNA molecules (Godine, Chin & Habener, 1980a), whilst the base sequences of the mRNA molecules regulating syntheses of the subunits are identical (Godine, Chin & Habener, 1982). With further elucidation of pathways of biosyntheses of gonadotropins at the molecular level in vivo, there is a tangible likelihood that research of the next decade shall unfold cost-effective processes for commercial synthesis of large quantities of homogenous LH and FSH subunits, and their assembly, in vitro

(Godine, Chin & Habener, 1980b; Strickland, Governman & Pierce, 1982). Developments of this kind shall quell major concerns regarding the treatment of subfertile patients with gonadotropins extracted from the urine of post-menopausal women.

2.2 Induction of endogenous gonadotropin secretion

Release of endogenous gonadotropins in mammals is controlled by gonadotropin-releasing hormone (GnRH). Since the structure of GnRH was established about 15 years ago (Matsuo et al., 1971), the molecule has been synthesized, as have a large number of synthetic GnRH agonists and antagonists (Karten & Rivier, 1986). Over the past 5 years, GnRH agonists and antagonists have been investigated principally with respect to their capacity to down-regulate endogenous release of gonadotropins, thereby allowing exogenous induction of ovulation without interference from endogenous gonadotropin release (Fleming et al., 1982). While these, and most other, studies on applications of GnRH (and its analogues) in the human have exploited the capacity of GnRH active substances to down-regulate pituitary function (Andreyko et al., 1987), appropriate regimes of treatment with GnRH and its agonists can activate pituitary secretion and restore a functional pituitary-gonadal axis (Ven, 1983). With regard to clinical concerns based on heterogeneity within families of FSH and LH molecules in gonadotropin preparations from human urine, particularly from urine of aged patients, advantages of inducing ovarian function in women through restoring endogenous pituitary gonadotropin secretion are self-evident. Ways in which GnRH (and GnRH agonists) might be used to correct disorders of fertility which indicate irregular patterns of endogenous GnRH secretion (ie.

hypothalamic amenorrhea; Reame et al., 1985) should therefore be further examined, and the scientific stage is set for such studies. Restitution and support of normal ovarian function in this way will also circumvent the problems associated with the use of urinary gonadotropin extracts, including the risk of adverse reactions to urinary gonadotropins (e.g. sensitivity to hMG, arterial thromboembolism, hemoperitoneum and birth defects). Investigations of the efficacy of controlling pituitary gonadotropin secretion with GnRH (or GnRH agonists) shall need to establish differences in biopotency of stored and releasable forms of gonadotropin within the human pituitary. Such differences have been demonstrated in other species (Mukhopadhyay et al., 1979) and appear to be attributable to different levels of incorporation of carbohydrate residues within the gonadotropin molecules, influenced by GnRH (Liu & Jackson, 1978).

2.3 Evaluation of gonadotropins

The content of gonadotropins in biological materials can be determined by radioimmunoassay, radioreceptor assay or by bioassay (in vitro or in vivo). In animal studies, ratios of receptor bound:immunoactive gonadotropin are highest in intact, mature subjects, compared with castrated or immature subjects. It has therefore been proposed that exposure of pituitary gonadotropes to gonadal factors stimulates the production of gonadotropin species with greater receptor affinity (Ulloa-Aguirre & Chappel, 1982; Solano, Garcia, Catt & Dufau, 1980). However, these same studies have established that not all immunoactive gonadotropin can bind to receptors, challenging quantitation of gonadotropins by immunoassay. Furthermore, determinations of bioactivity of LH

(e.g. stimulation of release of testosterone by cultured Leydig cells) and of FSH (e.g. stimulation of release of estradiol and/or plasminogen-activator from cultured granulosa cells) have shown that all receptor-bound gonadotropin is not bioactive (Wakabayashi, 1980; Ishii, 1980; Ulloa-Aguirre, Galle & Chappel, 1982; Lichtemberg, Weise, Schummacher & Wollring, 1982). Biological activity of the gonadotropins appears to be enhanced with reduction in sialic acid content of the molecules (Manjunath, Sairam & Sairam, 1982). Because subtle changes in numbers of sialic acid residues in gonadotropins need not affect immunoactivity or receptor binding, this finding supports other evidence for poor correlations between determinations of immunoactive, receptor-active and bioactive levels of gonadotropin in single samples (Dahl et al., 1986). While determinations of bioactive gonadotropin are biologically most meaningful, and bioassays are generally more sensitive than immuno or receptor assays, bioassays presently used for LH and FSH are more time-consuming and costly than immunoassays, and their applications are constrained accordingly. However, the evidence for heterogeneity within families of gonadotropins released by the pituitary (including variations in carbohydrate and sialic acid content) is consistent with divergence in results of immuno, receptor and bioassay of gonadotropin-containing materials and future research should therefore be directed toward development of rapid, accurate, precise and sensitive methods for determination of levels of biologically potent gonadotropins in biological materials.

Biopotency of gonadotropins used in the human can also be evaluated in sub-human primates, and a number of centers in the USA (including our own) are undertaking such studies. Results from these approaches should closely project responses by treated women, but in vivo bioassays are more time-consuming and more costly than in vitro bioassays, and could therefore be justified in few clinical applications.

2.4 Delivery of gonadotropins to induce gonadal function

Ovarian steroidogenesis and oogenesis in spontaneous cycles in women are supported by pulsatile changes in levels of gonadotropins, the frequency of release of LH and FSH during the cycle varying about 1 secretion every 1 to 2 h (Judd, 1985). For mainly practical (and cost) reasons, exogenous gonadotropins are usually administered as bolus injections, and are not often delivered in a pulsatile fashion. The biological effect of gonadotropins delivered by non-physiological routines is, therefore, likely to be compromised. Ongoing investigation of the nature and biological significance of pulsatile exposure of the ovary to gonadotropins (under control of GnRH; see Mais et al., 1986) during the ovulatory cycle of the woman, and during early pregnancy, shall establish ways in which modes of delivery of exogenous gonadotropins can more closely mimic those patterns of endogenous gonadotropin release which support development and release of a mature fertilizable oocyte, fertilization, nidation and pregnancy.

Preliminary studies shall need to establish the most appropriate bioactive gonadotropins to be used, and delivery systems should

then be tailored to mimic changes in bioactive gonadotropin levels in spontaneous cycles. By increasing the efficiency, and efficacy, of gonadotropin treatment of women during ovulation induction and through early pregnancy, it is likely that these developments shall markedly increase the success with which subfertility in women can be corrected.

2.5 Summary

Gonadotropin preparations have been used widely in the treatment of women with pituitary/ovarian dysfunction, and as hyperstimulatory agents in IVF patients. The experiences of many centers has highlighted deficiencies in the urinary gonadotropins presently used, and studies on the 'normal' hypothalamic-pituitary-gonadal axis have indicated that traditional regimen of administration of gonadotropins are likely to be suboptimal.

Research of the next decade must address these and other problems discussed in the overview above, and the research findings must be translated to provide infertile patients and their clinicians with efficacious methods of delivering gonadotropins of high biological potency, free of undesirable side effects.

3.0 Principles of Controlled Ovarian Hyperstimulation (COH) For Multiple Follicle Development During In Vitro Fertilization The prim centers substantiate the increasing odds of clinical pregnancy

occurrence by increasing the number of embryos utilized per transfer cycle (Edwards, 1983; Kerin, et al, 1984; Lopata, 1983; Hiller, 1985). Therefore, early in the menstrual cycle appropriate stimulation is utilized to recruit multiple follicles rather than allow selection and development of a single dominant follicle that will occur in the spontaneous, unstimulated ovarian cycle. The principles involved in ovarian stimulation are different in the IVF patient since these individuals have an intact hypothalamic-pituitary-ovarian axis producing normal ovulatory cycles. Prior to IVF, stimulatory agents had been primarily utilized in anovulatory individuals in order to produce a single healthy, mature follicle for ovulation. In the approach to the IVF patient, multiple follicles with synchronously developed oocytes is the desired result. This presents several areas of difficulty. First of all, in the intact ovulatory female, varying stages of follicle development and follicle maturation may be present at the initiation time of the stimulatory agent. Therefore, the resulting multiple follicle generation may have a very asynchronous maturation at the time of egg recovery. The actual numbers of follicles that can be generated in an individual cycle varies from patient to patient and within an individual patient it may vary from month to month. The stimulatory agents that are utilized will not select out a larger number of follicles than what the ovary has preselected, but instead, are merely a mechanism by which the selection process within the ovary is overridden. The optimal outcome would be to recruit the entire cohort of preselected follicles and mature them prior to collection for in vitro fertilization. The difficulty in this is that with the use of various agents for stimulation to

select and mature this cohort of follicles, adverse effects at the level of the oocyte as well as the endometrium may occur. In an earlier study from our institution we utilized a regimen of increasing gonadotropin stimulation in animals prior to in vivo mating or oocyte collection for in vitro fertilization. It was found that with both the in vivo produced embryos as well as the in vitro developed embryos, a direct negative effect from higher concentrations of gonadotropin stimulation was observed. Therefore it appears that the high levels of stimulation needed to produce multiple oocytes may have an adverse effect at the level of the oocyte prior to collection (Sato & Marrs, 1987). Moreover, the effect of high levels of estradiol and possibly other hormones may effect directly the endometrial synchrony during the stimulated cycle. Certainly endometrial development may have a major impact on clinical outcome during an IVF cycle (Edwards, et al, 1984). With the use of agents such as clomiphene citrate there may be even more of an adverse effect at the level of the endometrial lining since clomiphene does have antiestrogenic activities. However, this may be dose related in certain individuals and to date there have been no studies utilizing endometrial histology in cycles of controlled ovarian stimulation that have demonstrated clomiphene to have a direct adverse effect at the endometrial level.

Since the introduction of exogenous gonadotropins for multiple follicle recruitment during cycles of in vitro fertilization and embryo replacement (IVF-ER), significant advances in the management of controlled ovarian hyperstimulation (COH) have been

made by reproductive endocrinologists. However, marked variability in responses to exogenous gonadotropic agents has continued to confound efforts to tailor COH regimen to individual patients. Resultant aberrant folliculogenesis has been largely attributed to supraovarian origin and "medical hypophysectomy" with gonadotropin-releasing hormone (GnRH) antagonists before COH has been shown to reduce ovarian variability in responses of primates. Neveu and associates have recently reported on the improvement in ovarian response, fertilization rates and pregnancy rates after pituitary desensitization with Buserelin, a GnRH agonist (GnRHa), prior to the administration of exogenous gonadotropins. Further progress has been made with the use of pharmacological hypophysectomy using Leuprolide acetate in women who have demonstrated poor ovarian response to conventional COH. A comparative study was initiated at our Institution with analysis of 148 previous treatment cycles initiated with conventional COH in which only 26 (17.6%) oocyte collection cycles and 2 clinical pregnancies (7.7%) resulted. The introduction of leuprolide for down-regulation of the pituitary allowed oocyte collection in 25 of 27 previously treated women. Furthermore, nine clinical pregnancies were established (36%) in this extremely difficult group of women. The results of this preliminary study support the use of pharmacological hypophysectomy before the administration of exogenous gonadotropins for COH in women who have previously experienced inadequate ovarian responses to conventional COH. Moreover, our results concur with the hypothesis that supraovarian components significantly contribute to aberrant folliculogenesis in women undergoing COH. In addition, the evidence derived from the primate data with GnRH antagonistic analog, from the women

with severe hypogonadotropic hypogonadism and from the experiment of pharmacological hypophysectomy with leuprolide acetate, support the proposal that suppression of endogenous gonadotropin output improves the management of gonadotropin therapy in COH for in-vitro fertilization and embryo replacement.

Although, significant improvement has been accomplished in the past decade, the reproductive biologist still faces several problems with the management of COH, with the clinical use of GnRHa and conventional fertility drugs. The future is bright and the usage of GnRH antagonists without the stimulatory side effects of the agonists is almost a reality. Scientists of the Salk Institute (La Jolla, CA) and the University of California San Diego have recently utilized a potent antagonistic analog of GnRH (4F-antagonist) demonstrating a reduction of circulating bioactive endogenous gonadotropins after antagonist administration. Therefore, it is anticipated that 4F-antagonists and other antagonistic derivatives may be utilized in conjunction with exogenous gonadotropins in a more efficient manner. This therapy will reduce the initial stimulatory phase and the possibility of ovarian cyst formation observed with the GnRH agonists. Nonetheless, ovarian response to gonadotropins does not seem to be solely dependent on the hypothalamic-pituitary control. Certainly, the antral microenvironment rich in proteins, peptides and steroids play a significant role in the fate of the oocyte. Substances like inhibin, oocyte maturation inhibitor and follicle regulatory proteins contribute to the fine tuning and maturation of a healthy oocyte. It would appear therefore that research efforts in the next few years will be devoted to the area of the

antral microenvironment. New peripheral markers and probes of intraovarian regulators and more sophisticated ultrasound equipment with color resolution and magnetic resonance imaging systems will allow further understanding of the oocyte maturation process. This control of the physiologic mechanisms of hypothalamic-pituitary-ovarian regulation of follicle development and oocyte maturation will permit a more efficient production of healthier oocytes which is appropriately synchronized with the endometrial environment.

4.0 INVESTIGATIONS INTO MALE FACTOR INFERTILITY

When the infertile population is analyzed with respect to cause, it can be determined that pure female factors are responsible approximately 30% of the time, and pure male factors are causative in approximately 30% of couples. In the remaining couples, a combination of both female and male factors exists. Therefore, the male is involved in the infertile process approximately 50% of the time. It is important, however, to distinguish between infertile and subfertile males and females, and to realize that fertility potential is a relative phenomenon involving both partners. Many of the therapeutic approaches in reproductive medicine are directed toward enhancing fertility potential or gamete efficiency. This is particularly true of the new reproductive treatments and their role in the management of the subfertile male.

Under normal circumstances, the ejaculate is deposited high in the vagina at the time of intercourse. At the female's

midcycle, the cervical mucus is both abundant and receptive to the passage of spermatozoa. In addition, the aperture of the cervical is generally wider than at other times of the menstrual cycle. Shortly after being deposited, sperm begin migrating through the cervix and the remainder of the female reproductive tract in search of an oocyte. The interaction of sperm with cervical mucus is the possible initiating event of the CAPACITATION REACTION (Moore, et al, 1983) In general, the oocyte retains viability for 12 to 24 hours following ovulation, whereas sperm survive within the female reproductive system for approximately 24 to 48 hours after ejaculation. Fertilization is believed to occur high in the ampullary region of the fallopian tube.

The exact number of sperm required to initiate a pregnancy is unknown, but treatments such as IVFET, GIFT and IUI are particularly useful in that they bypass certain natural barriers of the female reproductive tract (cervix, uterus, fallopian tubes) and in so doing result in the closer approximation of gametes. Theoretically, these techniques are particularly useful in the treatment of the oligospermic or asthenospermic male whose sperm may not reach the ampullary region of the fallopian tube in sufficient numbers to initiate a pregnancy. Any such treatments, however, must be based on awareness that the natural mechanisms of SPERM CAPACITATION and the ACROSOME REACTION may be iatrogenically altered or physiologically impaired; thus in vitro sperm processing procedures must account for these changes so that when sperm are placed close to the oocyte they are capable of

fertilization. All treatments involving the "new reproductive technologies" utilize one of a variety of sperm processing media to prepare the sperm for insemination (Bavister, 1981). Ham's F-10 is a frequently used medium, either alone or in combination. It is a complex solution containing a variety of amino acids, vitamins and inorganic salts.

It is important to be aware of any intrinsic abnormalities of sperm function prior to the initiation of a treatment cycle. This may enable therapeutic sperm processing procedures to be tested ahead of time in an attempt to overcome the abnormalities and enhance the potential for fertilization.

4.1 Intrauterine Insemination (IUD)

This procedure involves the placement of large numbers of motile spermatozoa high in the fundus of the uterus at the appropriate time of the female's menstrual cycle (midcycle). The ejaculate is processed to separate the motile sperm from seminal plasma and cellular debris. Placement of the sperm high in the uterine fundus bypasses certain barriers of the female reproductive tract (most notably the cervix). IUI is used in couples with a cervical factor (cervicitis, cervical stenosis, inadequate mucus, hostile mucus), unexplained infertility, male factor infertility (specifically oligospermia or asthenospermia), and immunologic infertility (Allen, et al, 1985). Success rates vary between 8% and 10% per treatment cycle, and the outcome is highly dependent upon the initial indication for the treatment. The most favorable results are now reported to occur in couples with pure cervical factor infertility.

IVFET, GIFT and IUI are all established therapeutic modalities for certain infertile couples. There are several modifications of these procedures including direct intraperitoneal insemination (DIPI), transuterine tubal insemination (TUTI) and intrauterine fertilization (IUF). At the present time, these are considered somewhat experimental and their application in clinical practice must wait further trials.

In a significant number of subfertile males, defects of SPERM FUNCTION may coexist with abnormalities of sperm production. Even though their sperm may be placed in close proximity to the oocytes, the sperm cannot adequately penetrate the ZONA PELLUCIDA of the oocyte because of functional abnormalities. These patients are presumed to have defects in the mechanisms responsible for CAPACITATION and/or the ACROSOME REACTION.

4.2 THE SPERM PENETRATION ASSAY:

As noted in the discussion of the normal fertilization process, males with abnormalities of sperm production (oligospermia or asthenospermia) frequently have concomitant disorders of sperm function manifested as defective oocyte penetrability. Of great importance is the finding that abnormalities in sperm function may occur in some males despite normal bulk semen parameters (count, motility and morphology). In recent years it has become increasingly apparent that the functional competence of spermatozoa is not necessarily reflected by results obtained in the conventional semen analysis (Smith, et al, 1977).

In efforts to analyze the functional competence of spermatozoa, several laboratory tests have been developed. Three tests which have received critical attention are the zona free hamster egg sperm penetration assay (SPA), computer-assisted analysis of sperm movement, and measurement of sperm adenosine triphosphate (ATP) levels. Of these, only the SPA has had a significant impact on clinical management.

Species specificity of ovum fertilization is lost following enzymatic removal of the cumulus and zona pellucida from mouse, rat and hamster ova. Heterologous combination is of spermatozoa and zona-free oocyte from guinea pig, mouse, rat and hamster have demonstrated sperm chromatin decondensation within the egg cytoplasm and thus indicated that heterologous fusion had occurred (Hanada & Chang, 1972; Yanagimachi, 1972). Zona-free hamster oocytes demonstrate fusibility with spermatozoa from several species including the human (Banos & Ceal, 1982; Yanagimachi, 1981).

It is known that gamete membrane fusion occurs only if the spermatozoa have been capacitated and have undergone the acrosome reaction (Overstreet, et al, 1980), therefore, gamete fusion serves as a useful end point in determining the occurrence of these two phenomena. It has been demonstrated that heterologous fusion and the post fusion events of chromatin decondensation apparently do not differ from those in homologous fertilization (Banos, et al, 1979; Rudak, et al, 1978). Therefore, the use of this heterologous system provides an excellent model for diagnostic evaluation of sperm

function. Results from several laboratories indicate that the outcome of the SPA is highly correlated with results obtained when the test is performed in the standard fashion. In this way, information can be obtained regarding optimization of sperm function, and this information may be utilized in subsequent treatment cycles.

Factors which influence results of the SPA have been outlined by several authors (Johnson, et al, 1984; Rogers, et al, 1983; Syms, et al, 1985). It is important to emphasize, however, that there is no consensus on the specific protocol used for the SPA in the various laboratories now performing this test as a clinical assay. Therefore, valid comparison of results from different centers frequently is impossible. However, it is extremely important that for any given laboratory, the test be reproducible and sufficiently sensitive to avoid false negative results (failure to penetrate hamster oocytes but ability to penetrate human oocytes).

4.3 Future Considerations

The explosion of new information and the availability of therapeutic options has revealed new horizons in reproductive medicine. This certainly applies to the treatment of the subfertile male. As noted, IVFET, GIFT and IUI have all been successfully applied in male factor infertile couples with variable success. It should be emphasized that these techniques remain for the male who has failed routine surgical and medical treatment of known remediable causes of impaired testicular and/or sperm function. The therapeutic

applications of these newer treatment options have not been fully explored, and much remains to be learned about the pathophysiological mechanisms underlying disorders of male fertility; until these are better understood, many of the data we obtain from clinical investigations will be perplexing. It seems clear that adequate sperm production with normal bulk semen parameters is not necessarily associated with normal sperm fertilizing capacity. Likewise, although disorders of sperm production are often associated with functional abnormalities, they are not always present. This emphasizes the importance of evaluating sperm function in any couple in whom the male has not previously initiated a pregnancy. At the present time, the most reliable means of assessing sperm function is the SPA. Investigations into the applications of computer-assisted semen analysis are ongoing.

The scientific and technological advances relating to gamete accessibility and gamete handling has reached a level of sophistication which has provided researchers with a unique opportunity to perform investigations and undertake highly sophisticated therapeutic measures. For those individuals who suffer from defects of sperm function, several options either exist now or will likely become available in the near future. These include:

4.3.1 Methods to Improve Efficiency of Sperm

As has been implied, the fertility of certain males improved after sperm processing with human follicular fluid (hFF), Percoll gradient separation, albumin gradients, swim-up

procedures, TEST-yolk buffer co-incubation and pretreatment with a variety of other sperm capacitation media. As to whether these methods improve sperm efficiency directly (by having an effect upon capacitation or the acrosome reaction) or indirectly by changing the concentration of the motile fraction of sperm, is not clearly understood at the present time. Undoubtedly, additional methods of sperm processing will become available in the future, and an improved understanding of underlying sperm defects will facilitate application of the different therapeutic options with greater precision.

4.3.2 In Vitro Management of Oocytes

Much of the initial work from IVF centers focused attention on in vitro handling of oocytes by methods such as enzymatic or mechanical removal of cumulus cells and prolonged incubation of immature oocytes prior to the insemination. These studies demonstrated that the laboratory handling of the oocyte is crucial to fertilization and early embryo development (Marrs, et al, 1984). Studies pertaining to the optimization of the in vitro handling of oocytes (and the early embryo) are ongoing. The importance of this aspect of reproductive treatments involving IVFET and GIFT cannot be emphasized strongly enough. The future will improve our understanding of these phenomena still further. It is likely that a marginally subfertile male will not be able to fertilize oocytes under standard conditions, but if the oocytes (and the spermatozoa) are processed appropriately, fertilization may then occur.

4.3.3 Zona Drilling

This zona pellucida of the oocyte represents a barrier to the passage of sperm. The enzymes released from the acrosomal cap during the acrosome reaction are necessary to allow passage of the sperm through the zona and subsequent fusion of the sperm with the vitellin membrane of the oocyte (Gordon, 1987). For males with dysfunctional spermatozoa, acid drilling of the zona may allow passage of the sperm into the oocyte. As yet, no pregnancy has been reported following these procedures, and research in this area is ongoing.

4.3.4 Micromanipulation and Microinjection of Sperm

There is currently a great deal of research interest and effort in this area. Processed spermatozoa can be placed into the micropipette of a micromanipulator and injected directly into the ooplasm, thereby bypassing a potential dysfunctional mechanism of fertilization. At the present time, these techniques are experimental and concerns about possible genetic sequelae have been raised. Micromanipulation techniques will apply to several different aspects of reproductive medicine; however, it is anticipated that their major impact will be in the management of the infertile male suffering from disorders of fertilization.

4.3.5 Miscellaneous

Several other options are presently being investigated and may have clinical application for the subfertile male in the near future. These include:

Alloplastic Spermatocele

This option is for males with congenital absence of or damage of the vas deferens, in whom microsurgery has either failed or is not feasible. The design of the spermatocele and the ability to maintain viable sperm in an artificial medium for a prolonged period may improve so that the difficulties previously encountered with these techniques are overcome.

Direct Intraepididymal Aspiration of Sperm

This approach may become feasible for males with congenital absence of or damage to the epididymis and vas. As we understand more about sperm maturation process and are able to provide a suitable in vitro environment in which the final stages of sperm maturation may occur, patients so affected may become candidates for treatment.

Transuterine Tubal Insemination (TUTI)

This procedure is an extension of IUI in which the insemination catheter is placed transcervically into the uterus and through the cornual orifice of the fallopian tube. When perfected, this technique may be applicable for certain males with asthenospermia.

4.4 Summary

Although great strides have been made in recent years in the management of the infertile male, much remains to be

learned. Unquestionably, as our knowledge increases, other options will become available for these patients. With the greater accessibility of the gametes and early embryos and improved tissue culture techniques, the stage seems set for more sophisticated investigations to the mechanisms controlling reproductive processes. Molecular biologists and geneticists are making great progress in unraveling the genetic bases of many biologic phenomena and will undoubtedly uncover the genetic mechanisms controlling capacitation and the acrosome reaction in normal individuals. The result will be that our understanding of defects of these processes will broaden. Clinical application of genetic diagnostics and gene therapy seems imminent. Active research is vital to our acquisition of new knowledge. The application of this new knowledge to couples with male factor infertility will undoubtedly lead to higher pregnancy rates in the future.

5.0 Cryopreservation of Human Embryos

Successful cryopreservation of human embryos (Trounson & Mohr, 1983; Zeilmaker et al., Fehilly et al., 1985; Quinn & Kerin, 1986; Marrs, et al, 1987) helps overcome the wastage of "spare embryos" resulting from COH during in vitro fertilization (IVF) programs where the transfer of more than three embryos does not significantly improve pregnancy rates (Wood et al., 1985). It is also prudent to limit the number of embryos transferred per cycle in order to minimize the risk of multiple pregnancy (Kerin et al., 1983). Cryopreservation can also be used when problems arise due to adverse maternal conditions at the time of embryo transfer (Trounson & Mohr, 1983) and it allows for more

flexibility in the synchronization of cycles in an embryo/oocyte donation program (Lutjen et al., 1984). It has been suggested that decreased uterine receptivity is a sequela of ovarian hyperstimulation with clomiphene (Rogers, Milne & Trounson, 1984) due to the anti-estrogenic effect of clomiphene on estrogen receptors in the uterus. It has been suggested therefore that it may be more efficient if the embryos produced from a stimulated cycle are cryopreserved and subsequent transfer in a natural ovulatory cycle when the uterine environment may be expected to be more conducive for implantation (Zeilmaker et al., 1984). A marked degree of asynchrony occurs in the maturity of oocytes collected in cycles where hyperstimulation has been induced with human menopausal gonadotropin (hMG). Although immature oocytes at the germinal vesicle stage can be matured in vitro, fertilized and viable pregnancies result after transfer (Veeck, 1985), it is likely that the embryos resulting from such oocytes would be out of synchrony with the development of the uterus at the time of transfer. It may therefore be more appropriate to freeze such embryos for subsequent transfer in a natural ovulatory cycle.

The processes that need to be optimized during cryopreservation can be basically subdivided into three aspects. First, the events during cooling, second, storage in liquid nitrogen, and third, the process of thawing and revival of the cells.

When cells are cooled, the formation of intra-cellular ice crystals is lethal to the cells. The amount of water within the cells must be reduced so that when it freezes, the amount of intra-cellular ice formed is compatible with survival. If too much water is removed from the cells, they are excessively dehydrated and this is also

detrimental. Water containing ions and other solutes freezes at temperatures lower than 0°C depending on the concentration of the dissolved solutes. The greater the concentration of solutes, the lower the freezing point. As such a solution is cooled therefore, more and more pure water will freeze, and a greater liquid concentration of dissolved solute is left behind in solution. This increased osmotic pressure of the remaining solute can also cause damage to cells during cooling (the so called "solution effect"). The problem then is to remove sufficient water from cells compatible with their survival and to use a solution of such osmotic pressure that damaging solution effects do not occur.

No known biological activity occurs in cells frozen in liquid nitrogen and so they should be able to exist indefinitely under these conditions. The only types of reactions that take place at -196°C are photophysical, such as ionizing radiation. This type of radiation could cause genetic damage during storage at -196°C because the normal enzymatic repair mechanisms do not function at this temperature. Experiments with mouse embryos showed that background radiation over 200 to 1000 years would be required to cause enough genetic damage to appreciably reduce the survival of embryos stored in liquid nitrogen. It has been recommended that human oocytes and embryos are not stored for longer than the reproductive life of the women from which they were obtained or ten years.

Upon thawing, the reverse processes to those that apply during cooling operate. The basic aim is to rehydrate the cells and to remove any cryoprotectant which has permeated the cells. The rates of cooling and thawing are related to the amounts of intra-cellular ice remaining in the embryos when slow cooling is terminated at sub-zero temperatures by rapid plunging into liquid nitrogen.

Cryoprotectants act by a variety of mechanisms and more research needs to be done to gain a greater understanding of the action of various cryoprotectants. One of the main actions of cryoprotectants is to lower the freezing point of the solution. With an intra-cellular cryoprotectant such as 1.0 M DMSO, intra-cellular freezing may not occur until -40°C to -50°C . The cryoprotectants which have been used so far with human embryos are DMSO, glycerol, and propanediol, all intra-cellular cryoprotectants. The concentrations used have been between 1 and 2 M. When embryos are placed into solution containing these compounds, the cryoprotectant diffuses into the cells of the embryos at a rate characteristic to the membrane of the embryo at that particular stage of development. Water, however, crosses the cell membrane more readily than the cryoprotectant so that when the embryo is first placed in a solution of the cryoprotectant, it shrinks as water leaves the cell to dilute the high extracellular concentration of cryoprotectant. With time, osmotic equilibrium of the cryoprotectant will be achieved so that its concentration inside the cell will equal that outside; water then reenters the cell and it reswells to about its original size. Because of this shrinkage and reswelling of embryos when placed into solutions of permeable cryoprotectants, it is usual to add the cryoprotectant in a stepwise manner to minimize the degree of cell shrinkage. Calculations with bovine blastocysts indicate that these embryos can tolerate shrinkage to 0.4 of their isotonic volume. The rate of permeation of cryoprotectant and water is dependent on temperature, so that equilibrium will be achieved faster at higher temperatures. Cryoprotectants are usually added at room temperature.

When a solution is cooled the solidification of pure water (ice crystals) occurs, leaving behind the cell suspended in a hypertonic solution. However, in the case where small volumes are cooled at slow rates the phenomenon of super cooling can occur. Super cooled describes a solution which has been cooled to well below the freezing point without ice formation. When cryosolutions super cool, cells contained within do not dehydrate since extracellular ice has not been formed and the cells cannot respond as there is no increased osmotic pressure in the extracellular solution. Thus, to prevent super cooling ice crystal formation is induced (seeding) near the freezing point of the cryosolution. Seeding causes the cryosolution to become hypertonic to the cell due to ice formation. The cell can then respond osmotically to a hypertonic solution during the cooling process.

Since extracellular ice formation has been induced at seeding, the osmotic pressure of the extracellular solution increases as slow cooling proceeds as more ice crystals form and this leads to intracellular dehydration as water leaves the cell to balance the osmotic equilibrium with the extracellular solution. At some point, slow cooling is terminated by plunging the cells into liquid nitrogen. During slow cooling the cells respond by dehydrating and shrinking. The amount of water left within the cell to form intracellular ice on rapid plunging into liquid nitrogen depends on the degree of dehydration attained during the slow cooling process.

One of the main factors affecting the survival of frozen thawed embryos is the stage of development of the embryos at the time of cryopreservation. The human embryo appears to be similar to that of

the mouse in that all stages from one cell fertilized zygote to expanded blastocyst can be successfully frozen and thawed. There appears to be an interaction with the type of cryoprotectant used, however, which may reflect differences in permeability of the different stages to the different cryoprotectant. Overall results indicate that propanediol can be used satisfactorily for the one to four-cell stage, DMSO from the four to eight-cell stage, and glycerol can be used at the blastocyst stage. Perhaps the most difficult aspect of cryopreservation with IVF is the selection of embryos for fresh transfer versus those that are chosen for cryopreservation. There is a tendency to select the "best" embryos for transfer while relegating those which have little chance for initiating a pregnancy to cryopreservation. Currently, there are no methods available to indicate which embryos will initiate a pregnancy. The best data concerning embryo quality versus pregnancy rate come from the livestock embryo transfer industry. Here, data indicate that a minimum of 75% of fresh embryos recovered at the morula to blastocyst stage are capable of initiating pregnancies. Pregnancy rates after cryopreservation may range from 0% to 65% with an average of 50%. Thus, a little more than 50% of the best grade embryos are lost due to cryopreservation. Estimates of pregnancy rates per embryo transferred after in vitro fertilization in the human is 10-15%, that is, no more than 15% of cleaving embryos implant after transfer. Extrapolating from the livestock industry data, then, only 5 to 7% of the cryopreserved "best" grade human embryos can be expected to initiate pregnancies. Worldwide data is not available at present; however, some of the larger IVF programmes report pregnancy rates approaching 15% with multiple embryo transfers and 2-5% with single cryopreserved embryo transfers. These rates are improving as "better" quality embryos are selected for cryopreservation.

In the three most successful protocols reported in the literature, embryos are frozen at different times of development after insemination, viz; one to four-cell stage with propanediol, four to eight-cell stage with DMSO and at the blastocyst stage with glycerol. Since embryos show a decreased viability with increased periods of culture in vitro, a longer period of culture required for embryos to reach the blastocyst stage may act as a quality control to select only those embryos capable of continued growth. Whichever protocol is adopted the end result in terms of the number of pregnancies initiated as a proportion of embryos available for freezing at the time of transfer of the fresh embryos is somewhat similar. Although, only small numbers are available, it would appear as if the use of propanediol at the one to four-cell stage results in the greatest rate of pregnancy on a per embryo frozen basis. It would seem more appropriate to freeze embryos as soon as possible after insemination to minimize the adverse effects of in vitro culture.

5.1 Future Prospects

5.1.1 Techniques for Cryopreservation

New methods of freezing ova and embryos are currently being assessed (Trounson, 1986). We have obtained encouraging preliminary results with mouse embryos using the so-called 'ultra-rapid' freezing method. Because the method does not require a computer controlled freezing machine but merely involves placing the embryos in cryoprotective solution, packaging them into straws and then plunging in liquid nitrogen, it is likely that this method will find wide clinical application in most IVF programs. This, of course, depends on whether the method works equally as well with human embryos as it does with mouse embryos.

5.1.2 Clinical Aspects of Cryopreservation

The major application of cryopreservation in IVF technology will still be the storage of excess embryos produced from hyperstimulation in IVF and GIFT patients. These embryos will have been produced from the fertilization of the wife's ova by the husband's sperm. If the couple achieve a pregnancy from the transfer of fresh embryos, they may wish to utilize their cryopreserved embryos at a subsequent attempt at pregnancy without the necessity for another hyperstimulation cycle or they may wish to donate the frozen embryos to another infertile couple.

There will be a lesser, but still significant, demand for the cryopreservation of unfertilized human ova. The most pressing clinical application for this technique would be in the situation where a woman is facing the loss of her fertility due to pelvic disease, surgery, or imminent chemo and/or radiation therapy for ovarian cancer and other malignancies. She could have one or more cycles of ovarian hyperstimulation so that her oocytes could be collected and stored before she underwent treatment. Cryostorage of excess unfertilized ova from normal IVF and GIFT patients could also be offered to circumvent some of the legal and ethical problems inherent in the storage of excess embryos. It would also allow for greater flexibility in an oocyte donation program and could be used to conserve oocytes when the husband is unable to provide an adequate semen sample. There is no established technique for the cryopreservation of unfertilized human ova at present. We, and other groups, have some preliminary data indicating that up to

50% survival of human oocytes is possible after cryopreservation and up to 60% of the surviving ova subsequently undergo fertilization. Further studies are required in an animal model and with human ova donated specifically for research purposes to ensure that the cryopreservation process does not increase the incidence of chromosomal anomalies in embryos derived from cryopreserved ova.

5.1.3 Microinjection of Human Spermatozoa

Failure of human spermatozoa to fertilize ova when placed in contact with them as in the situation of IVF and GIFT could occur for several reasons. For example, there may be insufficient sperm with normal physiological function to achieve penetration of the cell and membrane barriers which surround the ovum. Alternatively, although the spermatozoa could be capable of fusing with the egg plasma membrane and becoming incorporated into the egg cytoplasm, but they are unable to bind to and penetrate the zona pellucida which is the membrane surrounding the ovum and which contains sperm receptors. The zona pellucida plays an essential role in preventing more than one sperm from penetrating the egg and is also important in ensuring the transport of the fertilized embryo from the site of fertilization in fallopian tubes down to the body of the uterus where implantation occurs. It has been suggested that mechanical breaching of the zona pellucida to allow spermatozoa to surmount this barrier could alleviate infertility due to the inability of spermatozoa to normally pass through the zona pellucida or where there are too few normal spermatozoa to achieve this.

Two techniques have been used with animal embryos (mouse, rat, hamster) to achieve fertilization in situations where otherwise it might not have occurred. These are a) direct insertion of spermatozoa into the ooplasm of the egg and b) placement of spermatozoa under the zona pellucida into the perivitelline space either by mechanical breaching of the zona or by the process of zona drilling using a micro-needle containing a medium of low pH (Gordon & Talansky, 1986). Acidified medium (pH 2-2.5) readily dissolves the zona pellucida of most mammalian ova without having a detrimental effect on the viability of the ovum. The second of these techniques viz placement of spermatozoa into the perivitelline space, is the one which has been used in the few attempts to apply this procedure to human gametes. In general, direct placement of spermatozoa into the ooplasm is less successful than perivitelline placement. Although not yet reported, it is likely that the incidence of abnormal spermatozoa fusing with the ova would be lower with perivitelline placement than if the spermatozoa are directly injected into the ooplasm. In the only reported data with human gametes where the spermatozoa were mechanically placed under the zona, 8 of 19 ova were fertilized. This included 5 of 7 mature oocytes and 3 of 12 immature oocytes which had been further matured in vitro. Two of these 12 immature ova were parthenogenetically activated by the procedure (Trounson, 1987).

It is likely that the zona drilling technique will have an important application in male infertility. It has already been shown to be a safe and effective technique in a mouse model

where normal young were derived from zona-drilled eggs (Gorden & Talansky, 1986). The technique would be particularly applicable to cases of infertility due to severe oligospermia, inability of the spermatozoa to penetrate the zona pellucida and in cases where frozen spermatozoa are used for fertilization since freezing damages some spermatozoa and a higher concentration of spermatozoa is required to achieve fertilization.

6.0 FACTORS CONTRIBUTING TO FUTURE RESEARCH ON TUBAL FUNCTION AND EXPLORATION

Although IVF and ET has overcome infertility due to irreversible tubal obstructive disease, and to some extent temporarily defocussed attention to on tubal disease, there is a major swing back to the tube, at both a basic research and clinical therapeutic level. There are important reasons for this. Firstly, the advent of complex and expensive procedures such as IVF and ET has made us take a more critical look at realistic pregnancy success rates following various types of tubal repair. Secondly, the increased frequency of sexually transmitted disease and its impact on the oviduct has made us aware of many organisms and their effect on these delicate structures. Thirdly, the development of gamete Intra Fallopian Transfer (GIFT) as a successful treatment of infertility in unexplained, endometriotic and male factor infertility encourages us to look at tubal implications of such processes. Fourthly, since IVF-ET procedures are complex and costly and associated, in most centers, with an overall successful pregnancy outcome per embryo transfer of 10 - 15%,

strategies for preventing, detecting and repairing tubal disease now takes a high priority. Fifthly, tubal factors which aid to improve the environment for early human development need to be explored.

6.1 Basic Research Questions Which Need to be Directed Towards the Oviduct

6.1.1 What is the precise mechanism by which the fimbria locate the oocyte and manipulate it into its lumen? What part do diseases like endometriosis, minor perifimbrial and periovarian adhesions play in compromising this process?

6.1.2 How critical is exposure of the oocyte, spermatozoa and early embryo to the oviductal environment for successful implantation? Are the success rates of IVF in part limited by lack of exposure to an oviductal environment? Early exposure of IVF produced monkey embryos to the tubal environment enhances subsequent development in vivo (Pauerstein, 1986). It has been demonstrated that the use of an IVF culture medium based on the composition of human oviductal fluid enhances the pregnancy rate following embryo transfer (Quinn, Kerin, and Warnes, 1985).

6.1.3 How critical is the synchronous transport of the oviductal embryo into the uterine environment? The timing of this event is critical to implantation in the rabbit but the answer to this question in the human remains unanswered and awaits schematic examination in the human or primate model.

6.1.4 How does the oviduct facilitate transport of the ovum from the uterus while at the same time, transport spermatozoa from the uterus towards the abdominal ostium? Are these mechanisms active, facilitative or mostly passive processes?

6.1.5 What are the precise interrelationships between the oviductal innervation, local transport and effect of steroids and prostaglanins on these processes under physiological conditions, during disease processes and following their resolution?

6.1.6 Why do humans develop tubal ectopic pregnancies? The answer to this question has major biological and clinical implications. The ramifications of tubal ectopic pregnancies are enormous in terms of a major risk to a woman's immediate health (it is still a life threatening entity, with a significant mortality rate, even in western countries with good health care systems) followed by long term infertility and emotional sequelae. The risk of ectopic pregnancy appears to be increasing following the widespread prevalence of sexually transmitted diseases (particularly from Chlamydial infection which is often subclinical and frequently produces minimal non obstructive endothelial damage predisposing to ectopic pregnancy), the use of intrauterine contraceptive devices, in relation to pelvic surgery, leading to peritubal damage and adhesion formation, restorative tubal surgery and tubal

anastomosis and following IVF and GIFT procedures. It is crucial that we learn to understand, and hopefully develop techniques for preventing the human embryo from implanting, when arrested in the oviduct. Tubal pregnancy is extremely rare in other primates and is nonexistent in infraprimates. If the embryo is deliberately arrested in the oviduct of infraprimates, it never develops beyond the blastocyst stage and dies. Is the human embryo or the human tube different in this respect from all other species?

6.1.7 How does minimal endometriosis affect fertility? It is still unclear whether endometriosis primarily interferes with ovulation, oocyte pick up, oocyte transport, spermatozoal survival, or embryo survival and transport. This disease is becoming relatively more common as women delay child bearing years into their mid to late thirties.

6.1.8 With global over population problems becoming a major issue in the next decade we need more simple, reliable, inexpensive methods of tubal sterilization which do not require anesthesia, are free from side effects and readily reversible. New technologies employing miniature fibre-optic systems and micro laser surgery techniques may make such achievements possible.

6.1.9 We need to define the regions of the oviduct which are necessary to optimize the success rate of tubal repair surgery. Will improved laser technology significantly improve upon present day surgical techniques.

6.1.10 The new technologies employing miniature tubal endoscopy called fallopscopy (Kerin, 1986) and the instrument called a falloposcope, incorporating small operating channels are currently in the process of being designed and the prototype instruments undergoing evaluation on the human fallopian tube both invitro and invivo. The successful application of such technology has major implication for overcoming infertility factors related to the oviduct. For example the techniques for trans-utero-tubal insemination (TUTI) of sperm (Kerin and Quinn, 1987), trans-utero-tubal gamete transfer, retrieval of tubal oocytes and embryos, and possible direct insemination of sperm into the tubal oocyte, are currently being developed.

6.2 LIMITATIONS TO OVIDUCTAL INVESTIGATION AND TREATMENT

The oviduct has particular properties which make it difficult to assess. Firstly, exploratory access to the medial aspect of the oviduct is via the cervical-uterine-route. Furthermore, the opening via the utero-tubal junction is only a millimeter or less in diameter. Secondly, access via the distal or fimbrial end can only be made via laparoscopy or laparoscopic techniques.

Direct endoscopic exploration of the outer third of the oviduct is now possible using the technique ampullosalpingoscopy (Cornier, 1986). Using flexible fiberscopes with diameters of 3-4 mm, exploration, diagnosis and limited treatments can be performed. However these techniques require laparotomy or laparoscopy and exploration is currently limited to the level of the ampullo-isthmic junction.

Preliminary studies for visualization of the inner portion of the oviduct from its medical aspect is now underway (Mencaglier et al, 1986). To date, however, adequate visualization of the inner tube has been difficult due to use of endoscopes which are too large, lack adequate flexibility, sufficient light transmission and resolving power. However, current advances in fiber optic and laser technology is likely to help us overcome these difficulties. The application of balloon angioplasty techniques to tubal strictures and obstructions is currently being explored (Confeiro et al, 1986) and with refinement, should provide useful clinical treatment for such disorders.

6.3 MINIATURE ENDOSCOPY OF THE FALLOPIAN TUBE: CURRENT DEVELOPMENT

In collaboration with the Laser Research Laboratory and Division of Cardiology at Cedars-Sinai Medical Center our division of Reproductive Endocrinology and Infertility has undertaken a study titled "Miniature Endoscopy of the Human Fallopian Tube (Falloscopy) : Feasibility Studies and Development of a Falloposcope". In summary, we have successfully developed a very fine flexible falloposcope measuring 1 meter in length and 0.6 mm external diameter. It has sufficient light transmission fibers and receiving fibers for adequate illumination and resolution of tubal structures. Because of its built in features, it is able to negotiate the narrowest part of the fallopian tube atraumatically. The falloposcope system has been coupled to a sophisticated video and recording system suitable for basic research study analysis and diagnostic recording.

The development and application of EXCIMER laser technology should permit us to utilize this unique source of energy via the falloposcope, since it has already been applied to the cardiovascular system (Grundfest et al, 1985). The advantage of excimer laser relates to the fact that it can be directed along very fine channels of 5-6 micrometers in diameter. Furthermore, excimer laser breaks molecular bonds using kinetic energy, not thermal energy. This property is most advantageous within the fallopian tube where it could be directed to divide intratubal adhesions, and related pathology without thermal damage to the immediate delicate tubal structures. Hopefully, such laser beams could be directed along fine operating channels of a falloposcope and utilized under direct vision. A list of possible application for an operating falloposcope follows:

6.3.1 Assessment of normal fallopian tube physiology

Cyclical endothelial changes

Cyclical vascular changes

Cyclical motility/functional sphincter changes

Cyclical neuronal innervation changes

Cyclical secretory changes

6.3.2 Assessment of normal tube/oocyte/spermatozoal/embryo physiology

Documentation of ovulation time and fimbrial movement.

Documentation of oocytes harvest, migration and fertilization.

Documentation of embryo transfer to the uterus via the utero-isthmic junction.

Studies and documentation of early implantation.

6.3.3 Assessment of fallopian tube pathology

Disorders of oocyte pick up by the fimbria.

Disorders of oocyte transport and fertilization.

Assessment of diagnosis and organism identification of tubal infection.

Assessment of microsurgical repair by end to end anastomosis, fimbriolysis and utero-cornual implantation.

Assessment of tubal endometriosis, secretory and macrophage populations of tubal fluid.

Diagnosis and lysis of intra-luminal adhesions.

Assessment of hydro or pyosalpinx development and drainage via the tubal ectopic pregnancy.

Early diagnosis and possible aspiration with hemostasis of tubal ectopic pregnancy.

Diagnosis and removal of microtubal polyps.

Assessment of hydro or pyosalpinx development and drainage via the tubal lumen.

Microbiopsy of tubal and extratubal structures.

6.3.4 Application of the falloposcope to treatment of tubal disease

Aspiration of tubal oocytes or embryos for in vitro culture or harvesting for assessment or possible donation to a recipient.

Intratubal insemination of oocytes (non-invasive gamete transfer technology).

Intratubal adhesiolysis and polypectomy and possible salpingolysis.

Follow up assessment of tubal infection, surgery and tubal ectopic pregnancy.

Transuterine obliteration of the interstitial portion of the fallopian tube as a reliable and simple means of sterilization.

Dilatation of tubal strictures, particularly those that commonly occur in the interstitial and medial portion of the fallopian tube following ascending infection from the uterus, utilizing angioscopic balloon catheters.

7.0 SURGICAL THERAPY IN REPRODUCTIVE MEDICINE

There have been major advances in surgical techniques and treatment of infertile patients in the past few decades. Newer suture materials with markedly decreased tissue reaction (and hence less post-operative adhesion formation), the development of microsurgical techniques and principles, a wider selection of antimicrobial agents, an improved understanding of the pathophysiology of adhesion formation coupled with development of pharmacologic agents to prevent and minimize adhesion formation, are but a few of the relatively recent major surgical advances applicable to our field. To consider that there will be no further advances in these specific areas in the coming years is undoubtedly wrong. One can only predict that these advances will be refined even further as future studies are completed.

Perhaps the major surgical advances that will occur in the next

decade will be the development of instrumentation which will enable many operations to be performed through a variety of endoscopes, thereby avoiding the need for major pelvic or vaginal surgery. The benefits of performing endoscopic surgery rather than a formal laparotomy are many and include major medical, financial, and social advantages for all concerned including patients, physicians, hospitals and insurance carriers. Because of this, one can only predict the major impetus of surgical research in the coming years will be toward developing and refining the techniques and instrumentation necessary to be able to operate in this fashion.

Table 1 summarizes a variety of medical conditions in our subspecialty which have heretofore been treated by major abdominal surgery. As a result of the development of new endoscopic instrumentation and the application of laser technology to these instruments, both the manner in which these procedures are performed, as well as the medical indications and modes of delivery of these treatments, will certainly undergo substantial changes in the coming years. While some of these changes have already started to take place in clinical practice, for the most part, these techniques are still in the developmental stages and major refinements are required. Table 2 lists a series of conditions for which it is predicated new instrumentation and technology will be applied in the future. The successful application of new instruments and technology will enable a series of innovative operations to be performed.

Another area of potentially fruitful research endeavor with potentially a reaching clinical application in our subspecialty, is the development of an artificial fallopian tube and/or tubal transplantation. While IVF-ET has resulted in a treatment modality which by-passes the fallopian tube, the treatment only has potential benefit in a given treatment cycle. Therefore, the successful creation of an artificial fallopian tube (or successful tubal transplantation), would allow conception to occur in any ovulatory cycle and possibly would greatly improve the chances of successful outcome in individuals with tubal disease. At the same time, this form of treatment would decrease the overall cost of treatment by avoiding the need for repetitive IVF-ET treatment cycles. In order for this endeavor to be successful, a great deal of research effort will be required into the physiology of tubal-ovum pick-up and tubal embryo transport mechanisms.

7.1 SUMMARY

It is becoming increasingly apparent that there is a major emphasis toward performing surgical procedures via endoscopes, thereby avoiding the need for major surgery with its attendant increased mobility and prolonged hospitalization. The advent and development of new instrumentation and the application of powerful laser technology to such instruments, has opened the way for performing operations quite differently from that which was formerly practiced. In addition, previously undescribed procedures are likely to be undertaken. There is much yet to be learned about the pathophysiological bases of many disease processes in our subspecialty. It is only with this additional knowledge that new treatments will be effectively developed and applied -- be they medical, surgical or a combination of both.

TABLE 1:

DISEASE	TREATMENT	DURATION OF HOSPITALIZATION	FUTURE METHOD OF TREATMENT	ADVANCE DUE TO	ADVANTAGE	DISADVANTAGE *
Ectopic Pregnancy	Laparotomy + Prior Laparoscopy	3-5 Days	Laparoscopic Resection, Laser Vaporization	Improved Endoscopic Instrumentation Laser Technology	- Avoids Laparotomy - Less Hospitalization - Less Adhesion Formation - Less Cost - Higher Pregnancy rates(?)	- Technical Difficulty/Hemorrhage May Require Laparotomy - Retained Trophoblastic Tissue-Require Repeat Operation.
Pelvic Adhesive Disease	Laparotomy + Prior Laparoscopy	3-5 Days	Laparoscopic Resection, Laser Vaporization	"	"	Inaccessability and extent of Disease Process. Requires Laparotomy.
Endometriosis	Laparotomy + Prior Laparoscopy	3-5 Days	Laser Vaporization	"	"	As Above and Injury to Surrounding Structure
Fibroids	Laparotomy Myomectomy	3-5 Days	Endoscopic Resection Laser Vaporization	"	"	Hemorrhage Necessitating Laparotomy
Ovarian Cyst	Ovarian Cystectomy ? Oophorectomy	3-5 Days	Laser Vaporization of Cyst Wall Lining	"	Avoid Laparotomy Less Hospitalization	- Uncertain Diagnosis - Recurrence - Possible Adhesion Formation
Menometrorrhagia	Total Abdominal vs Vaginal Hysterectomy	3-5 Days	Yag Endometrial Ablation	Yag Laser Hysteroscopic Techniques	- Avoid Laparotomy - Relatively Minor Procedure	- Uncertain Diagnosis - Recurrence - Pockets of Residual Endometrium unable to Diagnose Malignant Process.

*All Procedures Require Advanced and Specialized Training.

TABLE 2: PROJECTED FUTURE OPERATIONS IN REPRODUCTIVE MEDICINE

<u>DISEASE</u>	<u>OPERATION</u>	<u>INSTRUMENTS REQUIRED</u>	<u>ADVANTAGE</u>	<u>DISADVANTAGE</u>
.Intratubal Adhesions	Lysis of Adhesions	Falloposcope Salpingoscope Excimer Laser	Reconstitute Tubal Anatomy Without Major Surgery.	Development of Instrumentation.
.Tubal Endomet- riosis	Laser Vaporiza- tion			Limited Visualization Possible Future Ectopic Pregnancy
Tubal Stenosis or Occlusion	Tubal Dilatation	Tube Dilators	"	Same
Unexplained Infertility, Male Factor Infertility	Transtubal Aspiration of Oocytes and Insemi- nation	Falloposcope	Avoids Need for Laparoscopy	Same
Male Factor Infertility with Absence of Fertiliza- tion	Zona Pellu- cide Drilling, Oocyte Microinjec- tion	Micromanipu- lator +/- Applied Laser Technology	Ability to Treat Previously Untreatable Conditions	.Damage to Oocytes .? Genetic Sequelae
Infertility Amenable to Treatment With IVF-ET, Genetic Diseases, Sex Preselection	Embryo Splitting	Micromanipu- lator +/- Applied Laser Technology	.Greater No. Embryos for Genetic Analysis, Sex Selection or Cryopreservation .Improved Pregnancy Rates (?)	.Ethical Aspects of Embryo Surgery and Possible Embryo-Injury.

8.0 Psychological Evaluation of Participants in Assisted Reproduction

8.1. Stress

The adverse and disruptive effects of exposure to stress on fertility and reproductive functioning under extreme conditions are well established. The role of stress in affecting response to treatment in assisted reproduction is suspected but not established. Research will continue to investigate this area from the following standpoints:

8.1.1 Identifying sources of stress and quantifying reactions.

There has been insufficient study of the psychological effects of undergoing IVF and related techniques. While studies are available on the psychological characteristics of participants entering treatment (e.g. Haseltine et al., 1985; Freeman et al., 1985), there is little information on fluctuation of psychological state over the course of the treatment cycle. Such research will characterize sources of stress, quantify the reaction and identify moderator variables, such as social support, coping style, etc. The results obtained could be related to outcome variables, although owing to the many sources of variance, in combination with the skewed distribution of outcomes, such research would require large numbers.

8.1.2 Studying neuroendocrine correlates of stress

Exposure to stress is associated with a range of neuroendocrine changes, the most studied of which have been the release of catecholamines and cortisol. Psychological

research has related neuroendocrine changes to the pattern of release of catecholamines and cortisol. The subjective appraisal of the situation has been shown to influence the neuroendocrine response. For example, tasks associated with distress without effort (helplessness model) are associated with cortisol secretion, effort with distress is accompanied by secretion of both cortisol and catecholamines, while effort in the absence of distress with catecholamines release only (Frankenhauser, 1977). Concomitant study of psychological and neuroendocrine changes over the course of the treatment may shed light on the impact of stress on outcome. There is evidence that epinephrine may affect tubal function (see Stillman et al., 1986). The initial stages of implantation may be most vulnerable to the effects of stress.

8.1.3 Studying immunologic correlates of stress and conception

Recent studies have demonstrated the influence of psychological state on immune function (see Kiecolt-Glaser & Glaser, 1987). Exposure to stress has been shown to have both immune enhancing and suppressing effects, depending upon the parameters of the stressor under study (Shavit & Martin, 1987). Immunologic function will affect IVF outcome, in that pregnancy requires appropriate changes in immune function (Thurnau, 1985) and the relationship between psychological state, neuroendocrine function and immune function over the course of a treatment cycle and particularly post-transfer is an area requiring further study. In parallel, the effects of failed treatment on

immune function, as a function of the way in which this loss is appraised is also of interest because many participants undergo repeated treatment and an understanding of the enduring effects of failure may help to identify biochemical or immunologic markers that may, until resolved, reduce the likelihood of success on subsequent treatment cycles.

8.1.4 Using Laboratory Paradigms

Research will draw upon laboratory paradigms to investigate individual differences in psychological, physiologic and neuroendocrine response patterns to laboratory challenge. The assessment of reactivity to challenge draws upon the assumption that acute reactions observed in the laboratory setting may affect responses to challenges encountered in daily life. Initially research will describe the reactivity to standard laboratory stressors, as well as to stimuli of particular significance and emotional investment. This research may lead to the identification of certain patterns of reactivity, which, if triggered by treatment related stimuli, might indicate the benefits of undergoing desensitization approaches before participating in IVF and related treatment approaches.

8.2 Impact of Technology/Infertility

While the diagnostic and therapeutic benefits of technological advances continue to be studied, less attention has been paid to their psychological implications. Research will address the psychosocial implications of the new reproductive technologies.

Some of the questions to be addressed include:

8.2.1 Attitudes towards the various procedures and the physiologic and psychological toll taken by such techniques.

8.2.2. Impact of treatment on marital and sexual functioning

Research to date has tended to focus on the woman and neglect the male as well as the effects on the relationship. Ideally, participants will be assessed soon after their initial presentation for infertility evaluation and follow them over the course of subsequent treatments in order to describe the effects on the various aspects of their relationship and functioning. Such research needs to extend past the resolution of the problem (via assisted reproduction or adoption) or abandonment in order to provide longitudinal information over the entire time course. This research will draw upon chronic disease paradigms in terms of the intrusive and debilitating nature of the problem.

8.2.3. The effects of the problem on psychological state and in particular cognitive processing: Attention has been given to the relationship between information processing and mood from both theoretical (see Bower, 1981) and empirical standpoints (Teasdale, 1983). Characterizing cognitive changes associated with infertility and the way in which information on IVF and related techniques is processed, as

a function of mood and framing (Tversky and Turnerman, 1983) may help in understanding the cognitive distortions that occur in such patient populations. In a related vein, the infertile population provide an opportunity to study the nature of intrusive thoughts.

8.2.4. Studying the psychological reactions to pregnancy

The psychological reactions to becoming pregnant and the nature of attitudinal change taking place over the course of the pregnancy are of interest, along with anxiety levels and adherence to prenatal health care recommendations. This research effort will require the development of reliable and valid measures of maternal attachment to the fetus (see Reading et al., 1984). Changes of women conceiving through assisted reproduction can be compared with control groups conceiving naturally.

8.2.5 Post Treatment Adjustment

Although there have been advances in reproductive technology and they are expected to continue, most participants in such programs will not become pregnant. Studies are required on the psychological, social and interpersonal sequelae of failed participation. Such research will relate outcome to coping styles and other psychological resources assessed prior to treatment.

8.3 Research likely to lead to practical application for infertility prevention and treatment:

8.3.1 Screening and assessment of participants

It is necessary to develop a consensus on the role and format of psychological screening of participants in assisted reproduction programs. Research is necessary to delineate the variables of interest and to compile measures that possess psychometric rigour. These will both screen for psychopathology, as well as relate psychological state to outcome. The latter includes: advisability of undergoing treatment this cycle, biomedical outcome and psychosocial outcome. It is evident that existing scales, used in psychiatric screening programs, are of limited value in this context owing to the low base rates of cases, the item content and the defensiveness on the part of respondents, who are strongly motivated to avoid impediments to treatment. Measures need to be developed and related to program outcome criteria.

8.3.2 Implementation and evaluation studies of intervention programs aimed at reducing stress accompanying treatment in IVF programs: Interventions will consist of relaxation approaches alone or in combination with stress management techniques. This research can make use of random assignment to condition and dependent measures will include in addition to pregnancy outcome, the indices of psychological, interpersonal and marital adjustment.

8.3.3 Owing to the debilitating nature of infertility and the fact that a proportion of patients appear to become preoccupied with this problem in a self-defeating way, that

imposes strain both on themselves and their relationship, there is a need for research on the impact of interventions designed to offset these effects. The use of cognitive restructuring therapy (see Meichenbaum, 1985) may be of value, in terms of correcting the cycle of self defeating cognitions. According to models of depression, cognitions, whether primary or secondary, do seem to play an important role in identifying and maintaining a dysphoric state (see Beck, 1976). As a result, methods to help change these patterns may have a number of benefits; these may include: elevating mood and enhancing functioning, thereby limiting the compounding or additive effects of the cognitive distortions and dysphoria over and above the infertility; reducing stress by enabling the women to continue to function more effectively in other areas of life; enhance the feeling of control; and assist her in processing information more effectively, thereby enabling her to make rational and constructive decisions regarding future options etc. The latter may be significant because studies suggest impaired review of options and selection when under stress (see Janis Mann, 1977).

8.3.4 Evaluations of sexual functioning will describe the impact of assisted reproduction programs for sexuality. Of particular interest is the study of disorders of sexual desire. Research is needed on changes in sexual functioning over the course of infertility treatment.

SUMMARY

This report has attempted to look at the various areas of basic and clinical investigations that are currently ongoing or are possible in the future in the field of human reproduction.

The investigations into ovarian follicle activity and its effect on the central nervous system or on the oocyte itself is the key to moving more aggressively in the human reproductive tract in correcting ovarian disorders. The diagnostic and therapeutic problem in the male reproductive system are of equal importance. Moreover, the limited ability to cryopreserve gametes and/or embryos has wide spread impact not only on infertile couples but also on males or females who face a premature loss of gonadal function. The new instrumentation that is currently in the design stages will allow a direct viewing of the inside of the female (and possibly the male) reproductive tract but it also may allow a non-invasive approach for viewing early reproductive processes in vivo.

Certainly the ability to probe the human system is more available today as never before. Along with that ability comes problems with stress, fatigue, and emotional disturbances. These may play a major role in clinical outcome, thus the studies now and in the future, are vitally important in viewing the complete picture of the human reproductive process.

The areas discussed within this report are but a few of the investigations which will take place in the next 5 to 10 years. Where they lead us is not known. We do know, however, that our basic

knowledge of the human system will improve and thus our ability to treat these infertile couples will dramatically increase.

REFERENCES

- Adashi, E.T. Clomiphene citrate: mechanism(s) and site(s) of action - a hypothesis revisited. *Fertil Steril* 42:331, 1984.
- Aitken, R.J. Tubal and uterine secretions; the possibilities for contraceptive attack. *J. Reprod. Fert* 55:247-254, 1979.
- Alexander, D.C. & Miller, W.L. (1982) Regulation of ovine FSH chain mRNA by 17 α -estradiol in vivo and in vitro. *J. Biol. Chem.* 257:2282.
- Andreyko, J.L., Marshall, L.A., Dumesic, D.A. & Jaffe, R.B. (1987) Therapeutic uses of gonadotropin-releasing hormone analogs. Obstetrical and Gynecological Survey 42:1.
- Beck, A.T. Cognitive Therapy and the Emotional Disorders. International Universities Press, New York, 1976.
- Beyer, T.A., Rearich, J.I., Paulson, J.C., yrieels, J-P., Sadler, J.E. & Hill, R.L. (1979) Biosynthesis of mammalian glycoproteins. *J. Biol. Chem* 254:12531.
- Bower, G.H. Mood and Memory. *Am Psychol.* 1981, 36:129-148.
- Chappel, S.C., Ulloa-Aguirre, A. & Contifaris, C. (1983) Biosynthesis and secretion of follicle-stimulating hormone. Endocrine Reviews 4:179.
- Chen, C. Pregnancy after human oocyte cryopreservation. *Lancet* i:884, 1986.
- Confino, E., Friberg, J., Sleicher, N. Transcervical balloon tuboplasty. *Fert. Steril* 46(5):963-966, 1986.
- Cormier, E. Ampullosalpingoscopy. In: The fallopian tube: Basic studies and clinical contributions. 36, 383-390, Ed. M. Siegler, Pub. Futura Pub. Co., New York, 1986.
- Dahl, K.D., Pavlou, S.N., Kovacs, W.J. & Hsueh, A.J.W. (1986) The changing ratio of serum bioactive to immunoactive follicle-stimulating hormone in normal men following treatment with a potent gonadotropin releasing hormone antagonist. J. Clin. Endocr. Metab. 63:792.
- diZerega, G.S., Campeau, J.D., Nakamura, R.M. et al. Activity of a human follicular fluid protein(s) in spontaneous and induced ovarian cycles. *J. Clin. Endocrinol. Metab* 57:838, 1983.
- diZerega, G.S., Hodgen, G.D. Folliculogenesis in the primate ovarian cycle. *Endocrine Reviews* 2:27, 1981.
- diZerega, G.S., Marrs, R.P., Roche, P.C. et al. Identification of protein in pooled human follicular fluid which suppress follicular response to gonadotropins. *J. Clin. Endocrinol. Metab* 56:35, 1983.

- Edwards, R.G., Steptoe, P.C. Current status of in vitro fertilization and implantation of human embryos. *Lancet* II:1265, 1983.
- Fehilly, C.B., Cohen, J., Simons, R.F., Fishel, S.B., Edwards, R.G. Cryopreservation of cleaving embryos and expanded blastocysts in the human : a comparative study. *Fertil. Steril* 44:638, 1985.
- Filicori, M., Santoro, N., Merriam, G. et al. Characterization of the physiologic pattern of episodic gonadotropin secretion throughout the human menstrual cycle. *J. Clin. Endocrinol Metab* 62:1136, 1986.
- Fleming, R., Adam, A.H., Barlow, D.H., et al. (1982) A new systematic treatment for infertile women with abnormal hormone profiles. *Br. J. Obstet. Gynecol* 89:80.
- Frankenhauser, M. Job Demands, Health and Well Being. *J Psychosom Res.* 1977, 21:313-321.
- Freeman, E.W, Boxer, A.S, Rickels, K., et al. Psychological Evaluation and Support in a Program of in Vitro fertilization and Embryo Transfer. *Fert & Steril*, 1985, 43:48-53.
- Godine, J.E., Chin, W.W. & Habener, J.F. (1980) Luteinizing and follicle-stimulating hormones, cell-free translations of messenger RNA's coding for subunit precursors. *J. Biol. Chem.* 255:8780.
- Godine, J.E., Chin, W.W. & Habener, J.F. (1980) Cell-free synthesis and processing of the precursors of the subunits of LH. *J. Biol. Chem.* 256:2475.
- Godine, J.E., Chin, W.W. & Habener, J.F. (1982) subunit of rat pituitary glycoprotein hormones. Primary structure of the precursor determined from the nucleotide sequence of cloned cDNAs. *J. Biol. Chem.* 257:8368.
- Gordon, J.W., Talansky, B.E. Assisted fertilization by zona drilling: a mouse model for correction of oligospermia. *J. Expt. Zool* 239:347-354, 1986.
- Grundfest, W., Litvack, F.I., Goldenberg, T. et al. Pulseed ultraviolet laser and the potential for safe laser angioplasty. *The American Journal of Surgery* 15:220-226, 1985.
- Haseltine, F.P., Mazure, C., DeLaune, W., et al. Psychological Interviews in Screening Couples Undergoing in Vitro Fertilization. *Ann N.Y. Acad Sci.*, 1985, 442:504-522.
- Hillier, S.G., Afram, A.R.M., Margana, R.A. et al. Superovulation strategy before in vitro fertilization. *Clin. Ob. Gyn.* 12:687, 1985.
- Hodgen, G.D. The dominant ovarian follicle. *Fertil. Steril* 38:281, 1982.
- Ishii, S.H. (1980) Isolation and characterization of chicken follicle-stimulation hormone. *Gen. Comp. Endocrinol* 42:1.

- Istvan J. Stress, Anxiety and Birth Outcome. Psych Bull. 1986, 100:331-348.
- Janis, I., Mann, L. Decision Making. New York, Free Press, 1977.
- Judd, S.J. (1985) The neuroendocrinology of reproduction. In: Shearman, R.P. (ed) 'Clinical Reproductive Endocrinology'. Churchill Livingstone, New York, pp. 1-37.
- Karten, M.J. & Rivier, J.E. (1986) Gonadotropin-releasing hormone analog design. Structure-function studies toward the development of agonists and antagonists: Rationale and perspective. Endocrine Reviews 7:44.
- Kenigsberg, D., Littman, B.A., Williams, R.F. et al. Medical hypophysectomy: II. Variability of ovarian response to gonadotropin therapy. Fertil Steril 42:112, 1984.
- Kerin, J.F., Quinn, P. Washed intrauterine insemination in the treatment of oligospermic infertility. Seminars in Reproductive Endocrinology 5(1):23-33, 1987.
- Kerin, J.F., Marrs, R., Brand, E., Serafini, P., Quinn, P., Stone, B., Grundfest, W., Wade, M. Miniature endoscopy of the fallopian tube (Fallopscopy) : Submission approved by Human Subjects Committee, Cedars-Sinai Medical Center, February 1987.
- Kerin, J.F.P., Warnes, G.M., Quinn, P., Jeffrey, R., Kirby, C., Matthews, C.D., Seamark, R.F., Cox, L.W. Incidence of multiple pregnancy after in vitro fertilization and embryo transfer. Lancet 2:537, 1983.
- Kiecolt-Glaser, Glaser, R. Psychosocial Moderators of Immune Function. Annals Behav Med. 1987, 9:16-20.
- Knobil, E. The neuroendocrine control of the menstrual cycle. Recent Prog Horm res 36:53, 1980.
- Lassalle, P., testart, J., Renard, J.P. Human embryo features that influence the success of cryopreservation with the use of 1, 2-propanediol. Fertil. Steril 44:645, 1985.
- Lederman, R.P., Lederman, E., Work, B.A., McCann, D.C. The Relationship of Maternal Anxiety, Plasma Catecholamines and Plasma Cortisol to Progress in Labor. Amj Obstet & Gynecol. 1976, 132:495-500.
- Leibo, S.P., Mazur, P., Jackowski, S.C. Factors affecting survival of mouse embryos during freezing and thawing. Exp. Cell Res 89:79, 1974.
- Lichtemberg, V., Weise, H.C., Schummacher, M. & Wollring, C. (1982) Analysis of human pituitary LH isohormones by radioimmunoassay and different in vitro bioassays. Acta Endocrinol 99(suppl. 246):91.
- Liu, T. & Jackson, G.L. (1978) Modifications of luteinizing hormone biosynthesis by gonadotropin-releasing hormone, cycloheximide and actinomycin D. Endocrinology 103:1253.

- Lopata, A. Concepts in human in vitro fertilization and embryo transfer. *Fertil Steril* 40:289, 1983.
- Lutjen, P., Trounson, A., Leeton, J., Findlay, J., Wood, C., Renou, P. The establishment and maintenance of pregnancy using in vitro fertilization and embryo donation in a patient with primary ovarian failure. *Nature* 307:174-5, 1984.
- Lutjen, P., Trounson, A., Leeton, J., Findlay, J., Wood, C., Renou, P. The establishment and maintenance of pregnancy using in vitro fertilization and embryo donation in a patient with primary ovarian failure. *Nature* 307:174, 1984.
- Mais, V., Kazer, R.R., Cetal, N.S., Rivier, J., Vale, W. & Yen, S.S.C. (1986) The dependency of folliculogenesis and corpus luteum function on pulsatile gonadotropin secretion in cycling women using a gonadotropin-releasing hormone agonist as a probe. *Journal of Clin. Endocr. Metab* 62:1250.
- Manjunath, P., Sairam, M.R. & Sairam, J. (1982) Studies on pituitary follitropin. X. Biochemical, receptor-binding and immunological properties of deglycosylated ovine hormone. *Mol. Cell. Endocrinol* 28:125.
- March, C.M., Goebelsmann, U., Nakamura, R.M. et al. Roles of estradiol and progesterone in eliciting the midcycle luteinizing hormone and follicle-stimulating hormone surges. *J. Clin. Endocrinol. Metab* 49:507, 1979.
- Marrs, R.P., Lobo, R., Campeau, J.D. et al. Correlation of human follicular fluid inhibin activity with spontaneous and induced follicle maturation. *J. Clin. Endocrinol. Metab* 58:704, 1984.
- Matsuo, H., Baba, Y., Nair, R.M., et al. (1971) Structure of the porcine LH- and FSH-releasing hormone. I. The proposed amino acid sequence. *Biochem. Biophys. Res. Commun* 43:1334.
- Meichenbaum, D. Stress Innoculation Training. Pergamon Press, 1985.
- Mencaglia, L., Hanou, J., Perini, A., Cosmi, E. Transcervical and retrograde salpingoscopy: Evaluation of the fallopian tube in infertile patients. In: P The Fallopian Tube: Basic studies and clinical constrictions 35:377-381. Ed. M. Siegler. PTO
- Mohr, L.R., Trounson, A., Freeman, L. Deep-freezing and transfer of human embryos. *J. In Vitro Fert. Embryo Transfer* 2:1, 1985.
- Mukhopadhyay, A., Leidenberger, F., Lichtenberg, V. (1979) A comparison of bioactivity and immunoactivity of luteinizing hormones stored in and released in vitro from pituitary glands of rats under various gonadal states. *Endocrinology* 104:925.
- Nisell, H., Hjemdahl, P., Linde, B. Sympatho-adrenal and Cardiovascular Reactivity to Pregnancy Induced Hypertension: Responses to Isometric Exercise and a Cold Pressor test. *B.J. Obstet Gynecol.* 1985, 97:722

- Nisell, H., Hjemdahl, P., Linde, B., Biskow, C., Lunell, N. Sympatho-adrenal and Cardiovascular Responses to Mental Stress in Pregnancy Induced Hypertension. *Obstet & Gynecol.* 1986, 18:531-536.
- Pauerstein, C.J. In: *The Fallopian Tube, Forward*, VIII-IX, 1986. Ed. Siegler A.M. and Ansari A.H. Ed. Futura Pub. Co., New York.
- Paulson, R.J., Yee, B., Gorski, J., Laso, R.J., Marrs, R.P. Gonadotropin surges in the midcycle of hyperstimulated cycles after HCG administration (in prepatration).
- Pierce, J.G. & Parsons, T.F. (1981) Glycoprotein hormones: structure and function. *Ann. Rev. Biochem.* 50:465.
- Quinn, P., Kerin, J.F., Warnes, G.M. Improved pregnancy rate in human in vitro fertilization using a medium based on the composition of human tubal fluid. *Fertility and Sterility* 44:493-498, 1985.
- Quinn, P., Kerin, J.F.P. Experience with the cryopreservation of human embryos using the mouse as a model to establish successful techniques. *J. Vitro. Fert. Embryo Transfer* 3:40-5, 1986.
- Reading, A.E., Cox, D.N., Sledmere, C.M., Campbell, S. Psychological Changes over the course of Pregnancy: A Study of Attitudes Toward the Fetus/neonate. *Health Psychol.* 1986, 3:211-221.
- Reame, N.E., Sander, S.E., Case, G.D., Kelch, R.P. & Marshall, J.C. (1985) Pulsatile gonadotropin secretion in women with hypothalamic amenorrhea: Evidence that reduced frequency of gonadotropin-releasing hormone secretion is the mechanism of persistent evaluation. *J. Clin. Endocr. Metab* 61:851.
- Reichert, L.E. Lawson, G.M. (1973) Molecular weight relationships among the subunits of human glycoprotein hormones. *Endocrinology* 92:1034.
- Reichert, L.E. & Ward, D.N. (1974) On the isolation and characterization of the and subunits of human pituitary FSH. *Endocrinology* 84:655.
- Robertson, D.M., Van Damme, M.P., Diczfalusy, E. (1977) Biological and immunological characterization of human luteinizing hormones. I. Biological profile in pituitary and plasma samples after electrofocusing. *Mol. Cell. Endocrinol* 9:45.
- Robinson, J.O., Hibbard, B.M., Laurence, K.M. Anxiety During a Crisis: Emotional Effects of Screening for Neural Tube Defects. *J Psychosom Res.* 1986, 28:163-169.
- Rogers, P.A.W., Milne, B.J., Trounson, A.O. The effect of stimulation to produce multiple follicular development on embryo viability and uterine receptivity in human IVF. *proc. fertil. Soc. Aust.* 3:32, 1984.
- Roport, J.R., Quigley, M.E., Yen, S.S.C. Endogenous opiates modulate pulsatile LH release in humans. *J. Clin. Endocrinol. Metab* 52:583, 1981.

- Robert, J.R., Quigley, M.E., Yen, S.S.C. The dopaminergic inhibition of LH selection during the menstrual cycle. *Life Sci* 39:2067, 1984.
- Sato, F., Marrs, R.P. The effect of PMSG on mouse embryos fertilized in vivo or in vitro. *J. IVF-ET* 3:6, 1986.
- Shalev, E., Eran, A., Kerpel, S., Zuckerman, H. Psychogenic Stress in Women During Fetal Monitoring. *Acta Obstet Gynecol Scand.* 1985, 66:417-420.
- Shavit, Y., Martin, F.C. Opiates, Stress and Immunity: Animal Studies. *Annals Behav Med.* 1987, 9:11-15.
- Solano, A.R., Garcia, Vela, A., Catt, K.J. & Dufau, M.L. (1980) Modulation of serum and pituitary luteinizing hormone bioactivity by androgen in the rat. *Endocrinology* 106:1941.
- Stillman, R.J., Rosenberg, M.J., Sachs, B.P. Smoking and Reproduction. *Fert & Steril* 1986, 46:545-566.
- Strickland, T.W., Governman, J.M. & Pierce, J.G. (1982) Folding of the subunit of lutropin after in vitro biosynthesis. *Endocrinology* 110:198A.
- Teasdale, J. Negative Thinking in Depression. *Adv. Behav Res & Ther* 1983, 5:27-49.
- Thurnau, G. Reproductive Immunology. *Curr Probs in Obstet Gynecol Infertility* 1985, VII:No.6, 3-69.
- Trounson, A.O. Microfertilization. In: *Proceedings Fifth World Congress of IVF and ET, Norfolk, Virginia, 1987. Abstract No. PS-056.*
- Trounson, A.O., Mohr, L. Human pregnancy following cryopreservation, thawing and transfer of an eight-cell embryo. *Nature* 305:707-9, 1983.
- Tsafiri, A., Dehel, N., Bar-Ami, S: The role of oocyte maturation inhibitor in follicular regulation of oocyte maturation. *J. Reprod. Fert* 65:541, 1982.
- Tsoi, M.M., Hunter, M., Pearce, M., Chudleigh, P., Campbell, S. Ultrasound Scanning in Women With Raised Serum Alpha Fetoprotein: Short Term Psychological Effect. *J Psychosom Res.* 1987, 31:35-39.
- Tversky, Framing of Decisions and the Psychology of Choice. *Science*, Vol. 211; January 30, 1981, 453-458.
- Ulloa-Aguirre, A. & Chappel, S.C. (1982) Multiple species of follicle-stimulating hormone exist within the anterior pituitary gland of male golden hamsters. *J. Endocrinol* 95:257.
- Ulloa-Aguirre, A., Galle, P. & Chappel, S.C. (1982) Studies on the biological activity of each species of FSH within the anterior pituitary glands of male hamsters. *Endocrinology* 110:938A.

- Vargyas, J.M., Marrs, R.P. Endogenous luteinizing hormone release using human menopausal gonadotropins for in vitro fertilization. Accepted by J. IVF-ET, 1986.
- Veeck, L.L. Extracorporeal maturation. Norfolk 1984. Annal. NY. Acad. Sci. 442:357-67, 1985.
- Wakabayashi, K. (1980) Multiplicity of luteinizing hormone and its comparative aspects. In: Ishii, S. (ed). 'Hormones, Adaptation and Evolution'. Japan Sci. Soc. Press, Tokyo (Springer-Verlag, Berlin), pp. 271-279.
- Whittingham, D.G., Leibo, S.P., Mazur, P. Survival of mouse embryos frozen to -196°C and -269°C . Science 178:411, 1972.
- Wood, C., McMaster, R., Rennie, G., Trounson, A., Leeton, J. Factors influencing pregnancy rates following in vitro fertilization and embryo transfer. Fertil. Steril 43:245-50, 1985.
- Yen, S.S.C. (1983) Clinical applications of gonadotropin-releasing hormone and gonadotropin-releasing hormone analogs. Fertil Steril 39:257.
- Zaidi, A.A., Robertson, D.M., Diczfalussy, E. (1981) Studies on the biological and immunological properties of human follitropin: Profiles of two international reference preparations and of an aqueous extract of pituitary glands after electrofocusing. Acta Endocrinol 97:157.